UNIVERSITY OF ALBERTA

ROLE OF SPLEEN DURING EARLY PHASE OF LIPOPOLYSACCHARIDE-INDUCED HEMODYNAMIC PERTURBATIONS

BY

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DEPARTMENT OF PHYSIOLOGY

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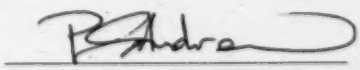

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DEDICATION

I would like to dedicate this thesis to the people who have done so much to help me reach this moment .... my father, Benjamin John Andrew, my mother, Jennifer Nantongo Andrew, my siblings, Susan, Christian, and John, and the love of my life, my partner, Sandra Mary Jerat.

I am sorry that you did not get to see this moment, Dad. Thank you so much for everything you have done for me over the last 29 years. I miss you and love you ... I will see you on the other side.

ABSTRACT

Within the spleen is a pathway for the efflux of protein-rich fluid from the intravascular compartment into the lymphatic system. Loss of such fluid can directly reduce plasma volume. Intrasplenic fluid efflux is enhanced by volume expansion, atrial distention, lipopolysaccharide (LPS), and the vasoactive factors adrenomedullin (ADM), atrial natriuretic factor (ANF), and nitric oxide (NO).

Septic shock, a leading cause of death in intensive care units throughout North America, is characterized by a severe and intractable hypotension coupled with a primary reduction in plasma volume. Animal models of septic shock, involving intravenous infusion of LPS to produce endotoxemia, have reported an LPS-induced decrease in plasma volume and blood pressure. The role that the spleen might play in these hemodynamic perturbations has not been investigated.

A non-lethal continuous low dose infusion of LPS in conscious male rats was used as a model for studying the role of the spleen in the hemodynamic response to endotoxemia. The pathway for intrasplenic fluid efflux could contribute to LPS-induced hypovolemia and hypotension. It was hypothesized that splenectomy, which would remove this pathway of fluid loss, should blunt these hemodynamic perturbations. Indeed, splenectomy did abolish the LPS-induced decrease in plasma volume and blood pressure.

Intrasplenic fluid efflux is driven by increased intrasplenic microvascular pressure (P_C), caused by differential vasoconstrictor tone of pre- versus post-capillary splenic resistance vessels. It was hypothesized that splenic sympathetic nerve activity, which increases during endotoxemia, normally limits LPS-induced intrasplenic fluid efflux. The results confirmed this, as splenic denervation exaggerated the intrasplenic fluid efflux, and worsened the hypovolemia and hypotension following LPS infusion.

It was proposed that differential vasoreactivity of splenic resistance arteries and veins to ADM, ANF, NO and endothelin-1 (ET-1), the plasma levels of which increase following LPS infusion, may contribute to the increase in intrasplenic P_C and subsequent intrasplenic fluid efflux. The *in vitro* results were consistent with this proposal.

In conclusion, fluid efflux from the splenic circulation of the rat contributes to the early LPS-induced decrease in plasma volume and blood pressure. This intrasplenic fluid efflux is regulated by nervous and hormonal inputs, which affect the tone of pre- and post-capillary splenic resistance vessels.

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LIST OF ABBREVIATIONS

ADM	Adrenomedullin
ANF	Atrial natriuretic factor
cGMP	Cyclic guanosine monophosphate
CLP	Cecal ligation and puncture
D-NMMA	N^G-monomethyl-D-arginine
ecNOS	Endothelial constitutive nitric oxide synthase
ET-1	Endothelin-1
GIT	Gastrointestinal tract
HEPES-PSS	4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered physiological salt solution
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
i.m.	Intramuscular
Inactin	Ethyl-(1-methyl-propyl)-malonyl-thio-urea
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
i.v.	Intravenous
ivc	Inferior vena cava
LBP	Lipopolysaccharide-binding protein
L-NMMA	N^G-monomethyl-L-arginine
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
ncNOS	Neuronal constitutive nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
P_A	Arterial pressure
P_C	Microvascular pressure
P_V	Venous pressure
PALS	Periarterial lymphatic sheath
PRA	Plasma renin activity
RSNA	Renal sympathetic nerve activity
s.c.	Subcutaneous
SNAP	S-Nitroso-N-acetyl-D,L-penicillamine
SNP	Sodium nitroprusside
Somnotol	Sodium pentobarbital
SSNA	Splenic sympathetic nerve activity
TPR	Total peripheral resistance
TNF-α	Tumor necrosis factor alpha
VSMCs	Vascular smooth muscle cells

CHAPTER 1.

INTRODUCTION

1.1 THE SPLEEN: AN OVERVIEW

The spleen is a highly vascularized organ that is situated in the left upper quadrant of the thorax (Fig. 1). Splenic tissue weight is approximately 0.05-1% of body weight, depending on the species (100). The human and rat spleen are anatomically and functionally similar (199). The different functions of the spleen are reflected in the different structural compartments that exist within this organ (100-102).

For many centuries the spleen failed to impress the scientific community as an organ worthy of investigation, given that removal of the spleen (splenectomy) or congenital asplenism did not appear to carry any physiological penalty (37). Galen (121-201 AD) referred to the spleen as an "organ of mystery" (56). Subsequently, van Leeuwenhoeck (1632-1732 AD) suggested a role for the spleen in the purification of blood (56). Later, the spleen was observed to be the organ with the largest aggregation of lymphoid tissue, and the only immune organ specialized for the filtration of blood (223). In more recent years, the immunological sequelae of splenectomy or congenital asplenism have been recognized (56,150). Consequently, both the hematological and immunological functions of the spleen are now established (150,220). However, evidence exists for other possible roles for the spleen: i) in the release of a hypotensive natriuretic factor (65,137), ii) as a peripheral organ involved in the regulation of blood pressure (136), and iii) as a pathway for iso-oncotic fluid shift from the intravascular compartment into the systemic lymphatic system (46,134,135). The underlying theme throughout the ensuing discussion will relate to the spleen as a pathway for iso-oncotic fluid shift from the intravascular compartment into the systemic lymphatic system, and how this phenomenon of intrasplenic fluid

extravasation may account for the early losses in plasma volume and fall in blood pressure associated with intravenous (i.v.) infusion of lipopolysaccharide (LPS).

1.1.1 Microvascular anatomy of the spleen

The spleen is part of the splanchnic circulation. The distribution of splanchnic arterial blood flow is divided among vascular beds supporting the spleen, liver and mesentery (Fig. 2). The splenic (or lienic) artery, a branch of the celiac artery, is the source of blood to the spleen. This vessel divides many times into what are termed hilar arteries that, at approximately 200μm in diameter, influence splenic vascular resistance (Fig. 1). Hilar arteries pierce the capsule of the spleen, and then divide further into trabecular arteries. These vessels, which eventually enter the pulp of the spleen as central arteries, structurally resemble small arterioles or capillaries. Venous drainage of the spleen begins in the red pulp, in venous sinuses. Some arterioles terminate close to these venous sinuses, whilst functional arteriovenous shunts within the spleen may be opened to enable the fast passage of blood through the spleen. The venous sinuses drain into trabecular veins, which then go to the hilus where blood leaves via hilar veins that feed into the splenic vein. The splenic vein feds into the portal vein.

1.1.2 Blood flow to the spleen

Blood flow to the spleen is extremely high, with resting splenic blood flow being between 40 – 100 ml/min per 100 g of tissue weight; up to 10% of cardiac output (100,102). In the conscious rat, splenic blood flow is approximately 8 ml/min (46). Under euvolemic conditions, about 25% of cell-free fluid flowing into the spleen is removed

from the circulating blood (46). This shift of iso-oncotic fluid from the intravascular compartment into the systemic lymphatic system thus influences circulating blood volume. Given the high rate of blood flow, the spleen has great potential to control changes in circulating blood volume.

The structure of the spleen is such that the route taken by blood through the splenic circulation may be altered according to the state of volemia of the animal (46,103,145,187,267) (Fig. 3). Under euvolemic conditions approximately 90% of blood going into the spleen and out via the splenic vein, travels via so-called "fast pathways" with a red blood cell (RBC) washout half-time of ~30 seconds (53,102). The remaining 10% of total blood flow travels through slow and intermediate pathways of the red pulp (100,103,223) with a RBC washout half-time of 8-54 minutes (53,102). Under conditions of hypovolemia, the total blood passing through fast pathways containing arterial-venous shunts increases to almost 100% (53,102,103). However, in hypervolemia, blood takes a slow, tortuous path through the red pulp of the spleen (145).

There are numerous autocrine, paracrine, and endocrine agents that can affect splenic blood flow. Interleukin-1 (IL-1), one of the first mediators released by the host in response to LPS, increases splenic blood flow via inhibition of the sympathetic vasoconstrictor tone in the rat spleen (203). Nitric oxide (NO) is another vasoactive agent that has been reported to affect splenic blood flow (10). Thus, the vasodilatory effects of such hormones as adrenomedullin (ADM) which increase NO biosynthesis and whose plasma levels may increase 40-fold during sepsis (114,183), cannot be overlooked with

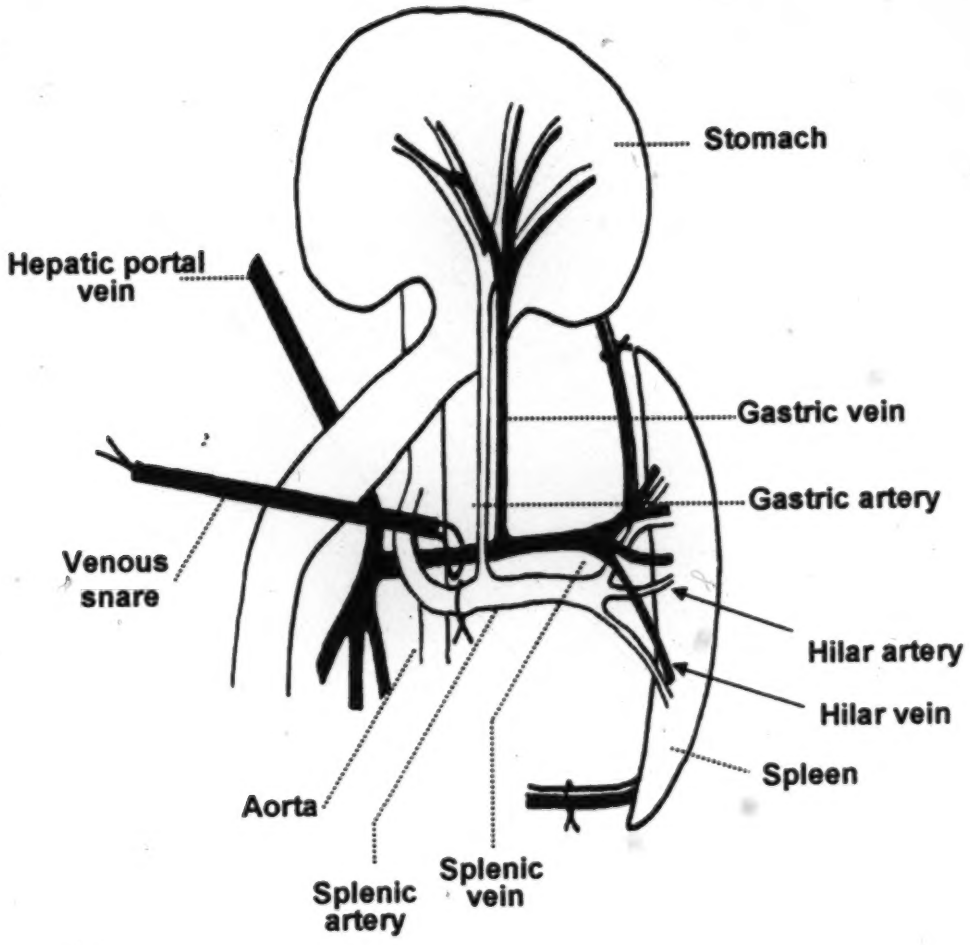

Figure 1. The vasculature of the spleen. Illustration adapted from Chen and Kaufman (1996) (46).

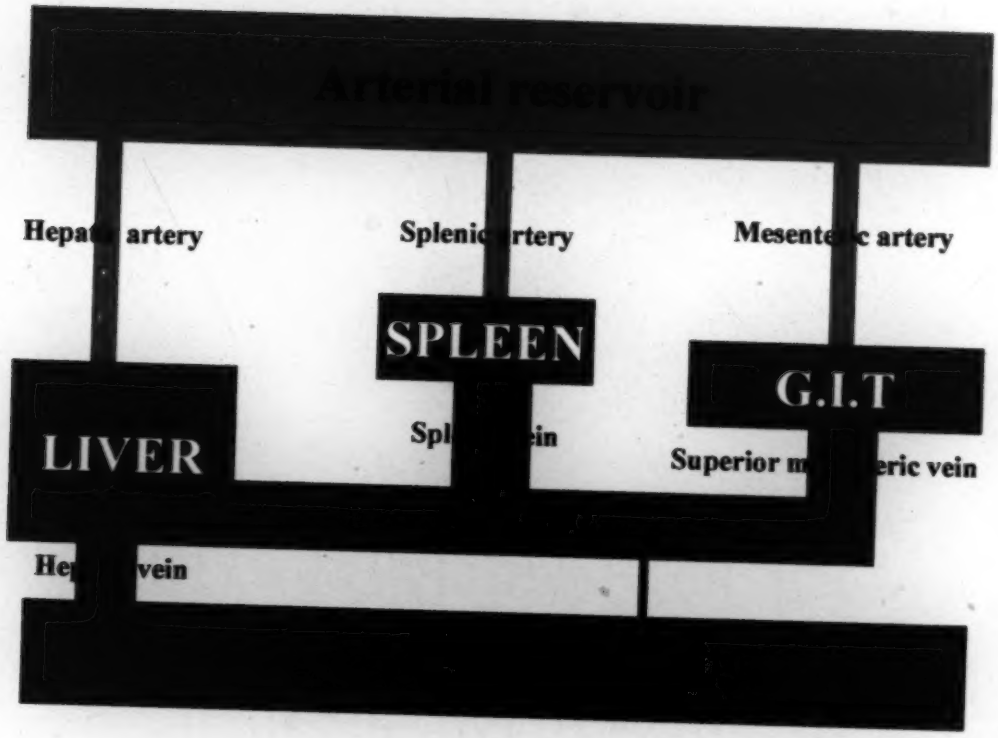

Figure 2. A simplified view of the splanchnic circulation. Reproduced from Isbister (1997) (119).

regards to their possible effects on splenic blood flow (110). In summary, control of splenic blood flow is influenced by the interaction of many inputs, both hormonal and neural. These alter the hemodynamic forces within the splenic circulation, at least in part, through their effect on the vascular tone of the splenic resistance vessels.

1.1.3 Nerve supply to and from the spleen

The splenic nerve carries both afferent and efferent neural pathways. With regards to the efferent nerves, sympathetic (adrenergic) fibers predominate with little, if any, parasympathetic innervation (22). There is evidence for baroreflex control of splenic nerve activation (23,24,47,199), and for an increase in splenic sympathetic nerve activity (SSNA) in response to endotoxin (157). Sympathetic vasoconstrictor fibers are directed primarily to the splenic arterioles rather than venules (3,22,180). These sympathetic vasoconstrictor fibers follow the course of the splenic artery and provide smooth muscle control over the branching resistance vessels. Hence, increased SSNA would cause an increase in the tone of pre-capillary vessels compared to post-capillary vessels. Consequently, increased SSNA would be anticipated to lower intrasplenic microvascular pressure (P_C).

In addition to the sympathetic vasoconstrictor fibers within the splenic nerve (3,22,180), it has been established that there are, despite one report to the contrary (180), sensory afferent fibers (48,152,164,165,252). Therefore splenic denervation would, in addition to removing the splenic sympathetic nerves (which would predominantly affect the vascular tone of pre-capillary splenic resistance vessels), also remove sensory afferent

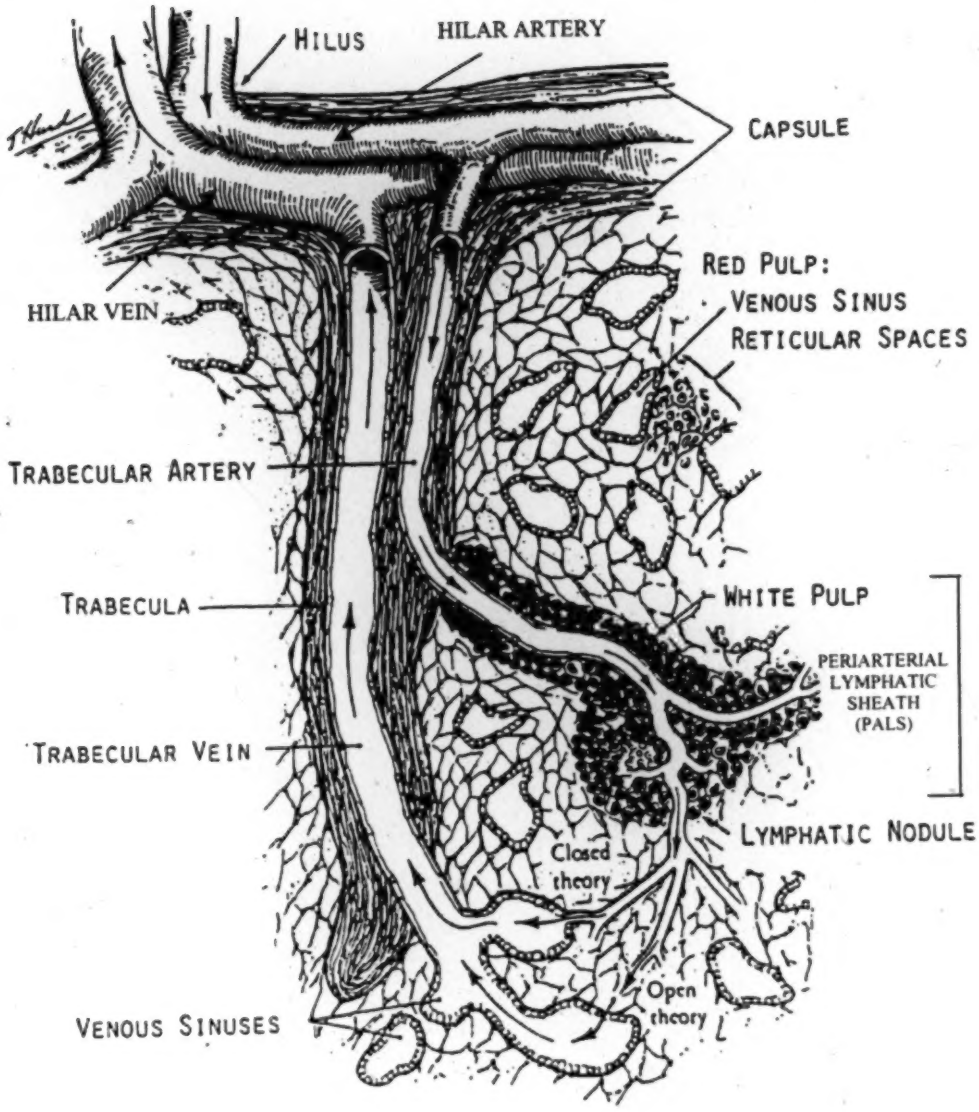

Figure 3. Schematic illustration of the sinusoidal spleen of a rat. Illustration adapted from Groom and Schmidt (1990) (101).

innervation. These sensory afferents have been proposed to form part of a neural reflex pathway between the spleen and kidney termed a spinal splenorenal reflex (164,165,252), whereby sensory afferent traffic from the spleen inhibits renal sympathetic nerve activity (RSNA). Hence, removal of this inhibitory action of the spinal splenorenal reflex (by splenic denervation) would be anticipated to elevate RSNA which, in turn, would affect renal function, PRA, and blood volume (172).

1.2 CONTROL OF BLOOD PRESSURE UNDER NORMAL PHYSIOLOGICAL CONDITIONS

Homeostasis of mean arterial pressure (MAP) is achieved through the actions of a number of interacting regulatory mechanisms that function in a manner to compensate for changes in cardiac output, total peripheral resistance (TPR) and blood volume (58) (Fig. 4). Each of these mechanisms is geared towards maintenance of MAP in the face of moment-to-moment and long-term changes in MAP (58,201).

1.3 FLUID SHIFTS WITHIN THE MICROCIRCULATION

The forces (generally referred to as the Starling forces) that determine fluid exchange at the capillary are the capillary hydrostatic pressure, interstitial fluid hydrostatic pressure, capillary colloid osmotic pressure and the interstitial colloid osmotic pressure (Fig. 5). Of these four factors that influence fluid movement across a capillary, it is the capillary hydrostatic pressure that is most subject to homeostatic regulation, via alterations in pre- versus post-capillary vascular tone. The control of fluid movements across capillaries is an important component in the regulation of intravascular volume.

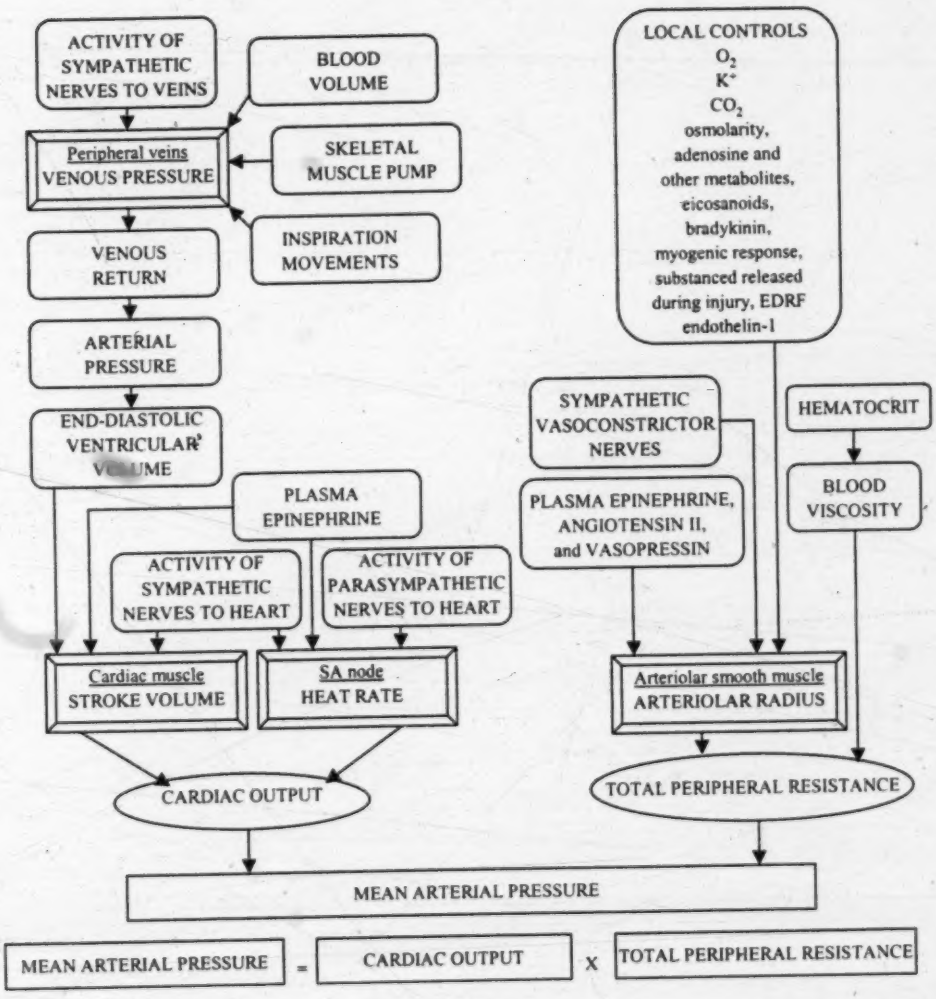

Figure 4. Simplified overview of the maintenance of a constant mean arterial pressure by nervous and hormonal mechanisms. Reproduced from Rhoades and Pflanzer (1996) (201).

In most vascular beds, the endothelial barrier prevents the free movement of macromolecules (such as albumin) across the microvascular endothelium. However, the endothelial barrier is not completely impermeable to large molecules. The movement of large macromolecules from the intravascular compartment into interstitium, although significant but extremely small, occurs across capillaries lined mostly by continuous endothelium via specialized transport pathways (168,202). However, in tissues such as the spleen that possess a discontinuous vascular endothelium the movement of macromolecules can readily occur. For example, the rate of protein extravasation is particularly high in organs like the spleen (202), an organ that is known to have a discontinuous vascular endothelium (232).

The concept of "third space" shifting proposes that fluid shifts from the intravascular to extravascular space arise when there is an alteration in the Starling forces (79,239). For example, during tissue inflammation, the colloid osmotic imbalance caused by the leakage of plasma proteins into the extravascular space leads to third space fluid shifts (79). The third space has been defined as " ...that reservoir which ordinarily would act as a second space or that would restore volume to a depleted vascular space secondary to the fall of hydrostatic pressure created by volume depletion" (79). In a pathophysiological state such as endotoxemia where there is capillary damage, fluid losses from the intravascular compartment cannot be restored because of an increased interstitial colloid osmotic pressure and decreased plasma colloid osmotic pressure, since this imbalance favors diffusion of fluid out of intravascular into extravascular tissues (79,239). The loss

of protein-rich fluid from the intravascular compartment can thus influence the homeostatic maintenance of plasma volume and, ultimately, blood pressure.

1.3.1 Fluid extravasation from the splenic circulation

There is ample evidence suggesting a role for the spleen in the regulation of plasma volume (46,134,135). Briefly, these studies suggest that, within the spleen, there is a gateway for extravasation (or shift) of protein-rich fluid from the intravascular compartment into the systemic lymphatic system. Loss of fluid that is iso-oncotic to plasma stands to cause a direct fall in plasma volume status. Intrasplenic fluid efflux is enhanced by volume expansion, atrial distension, LPS, atrial natriuretic factor (ANF), and NO (7,8,134,228).

Within the spleen, there is a large flow of lymph from the red pulp to the deep lymphatic vessels in the periarterial lymphoid sheaths (PALS) (193,211) (Fig. 3). The flow of lymph runs countercurrent to the direction of arterial blood flow, and it is in the region of the white pulp that immune cells are activated and disseminated to the rest of the body via splenic lymphatic drainage (33,124,211).

Evidence for changes in intrasplenic P_C and subsequent increases in fluid efflux from the splenic circulation has been reported in an anesthetized rat preparation of a blood-perfused spleen (8,228). Intrasplenic infusion of ANF and NO increases post- versus pre-capillary resistance, elevates intrasplenic P_C and increases fluid efflux (228), plus intrasplenic infusion of NO elevates intrasplenic P_C and fluid efflux from the splenic

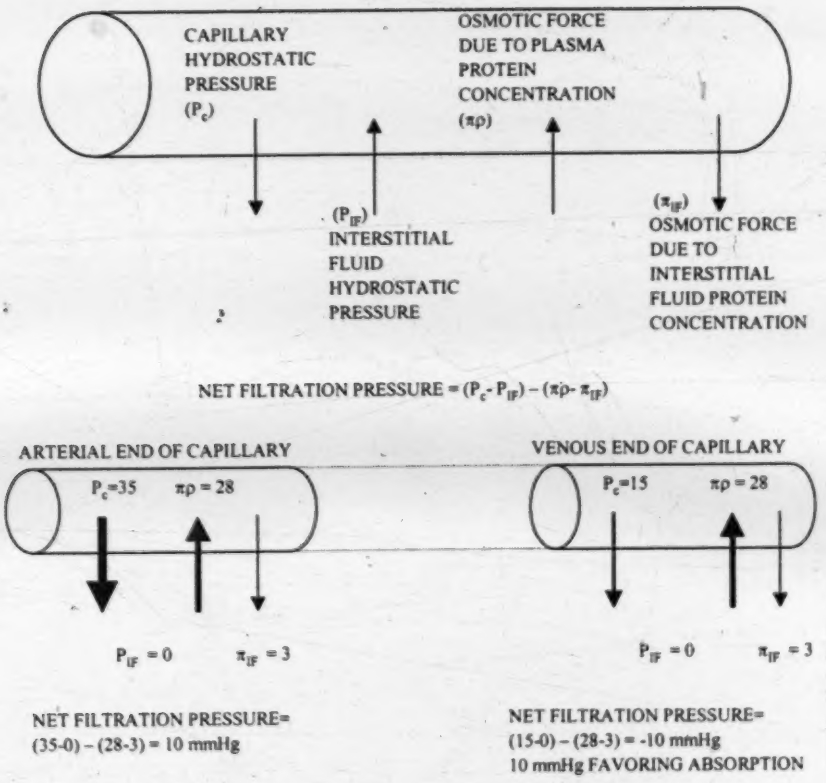
CAPILLARY HYDROSTATIC PRESSURE (P_c)
OSMOTIC FORCE DUE TO PLASMA PROTEIN CONCENTRATION ($\pi\rho$)
(P_{IF}) INTERSTITIAL FLUID HYDROSTATIC PRESSURE
(π_{IF}) OSMOTIC FORCE DUE TO INTERSTITIAL FLUID PROTEIN CONCENTRATION
NET FILTRATION PRESSURE = $(P_c - P_{IF}) - (\pi\rho - \pi_{IF})$
ARTERIAL END OF CAPILLARY
P_c=35 $\pi\rho$ = 28
P_{IF} = 0 π_{IF} = 3
NET FILTRATION PRESSURE= (35-0) – (28-3) = 10 mmHg
VENOUS END OF CAPILLARY
P_c=15 $\pi\rho$ = 28
P_{IF} = 0 π_{IF} = 3
NET FILTRATION PRESSURE= (15-0) – (28-3) = -10 mmHg
10 mmHg FAVORING ABSORPTION

Figure 5. The Starling Equilibrium: factors determining fluid movement across capillaries. Reproduced from Rhoades and Pflanzer (1996) (201).

circulation (8).

Fluid extravasation from the splenic circulation of the rat is surprisingly large considering the plasma volume of this species. The reported intrasplenic fluid efflux in conscious unrestrained rats is approximately 0.2 ml/min/100g body weight (46). Basal intrasplenic P_C in the anesthetized rat is approximately 12 mmHg (8,228). The significance of fluid extravasation from the splenic circulation can be contrasted with the known importance that the kidney has in the control of plasma volume. In the rat kidney, glomerular filtration rate is approximately 1 ml/min/100g body weight (21) with a glomerular filtration pressure of approximately 46 mmHg (237). The kidney is the major organ for control of the total extracellular fluid volume. However, because it is impermeable to macromolecules such as albumin (34,200), the kidney is not capable of specifically regulating intravascular protein content and plasma volume. By contrast, ultrafiltration of iso-oncotic fluid from the splenic circulation can directly reduce plasma volume and, ultimately, blood pressure.

1.3.2 Tone of pre- versus post-capillary splenic resistance vessels

The vasoconstrictor action of SSNA (predominantly on pre-capillary vessels) would be tempered by the opposing or complimentary actions of local and circulating vasoactive agents. For example, the vasoconstrictor effect of increased SSNA on splenic resistance arteries would be expected to oppose the vasorelaxant actions of NO release within the splenic microcirculation. Thus, the tone of splenic resistance arteries and veins will depend on the balance between nervous inputs, circulating and local vasoactive factors.

Moreover, the balance between vasodilatation and vasoconstriction of the splenic resistance vessels will influence the contribution of the splenic vascular bed to TPR. In addition to this complex interaction between neural inputs and vasoactive agents, there is the possibility of differential vasoreactivity of splenic resistance arteries and veins to vasoactive agents. This has been suggested to explain the divergent responses (both quantitative and qualitative) of different vessels within a single vascular bed to a common vasoactive stimulus (12,55).

The Starling equilibrium describes the factors controlling movement of fluid across all vascular beds, including those within the spleen (Fig. 5). Pre-/post-capillary changes in vascular tone largely determine capillary filtration pressure (16,17,233). Through a mechanism resembling that found in the renal microvasculature (where differential vasoreactivity of afferent and efferent glomerular arterioles to vasoactive agents causes changes in glomerular filtration pressure) (121), the differential vascular tone of splenic resistance arteries (termed hilar arteries) versus veins (termed hilar veins) determines the intrasplenic P_C , which is the driving force for intrasplenic fluid efflux (228). Thus, the intrasplenic extravasation of protein-rich fluid is not due to alterations in capillary permeability, given that the splenic microvasculature is freely permeable to cell-free fluid, but is due to a rise in intrasplenic P_C.

1.4 SHOCK: AN OVERVIEW

Jacobson (1968) proposed a physiological approach to the definition of shock, and suggested that this condition be defined as "....acute circulatory insufficiency

characterized by cardiac output inadequate to provide normal perfusion for the major organs" (123). At the time, clinical definitions considered shock as a sudden development of abnormally low arterial pressure. Such a definition was criticized as not being a representative reflection of the cause of the shock (123). Disapproval of this clinical definition for shock was also based on the fact that circumstances may exist where compensatory mechanisms are still maintaining blood pressure (Fig. 7), but such a definition obscures the fact that there may be an abnormal cardiac output or TPR. Other attempts at defining shock have been based on etiology or function (138). An etiological classification defines shock in terms of hypovolemic, cardiogenic, septic, neurogenic, allergic, obstructive, and endocrine-drug causes. A functional classification considers shock in terms of hypovolemic, cardiogenic, obstructive, and distributive events. Ultimately, although there appears to be no all-inclusive definition of shock it is recognized that the end result of the many different forms of shock is essentially the same, namely, progression to a state where circulating blood volume is insufficient to ensure adequate tissue perfusion.

Shock is characterized by a series of stages identified by particular hemodynamic features (15,123). The *early* stage is temporary and is characterized by compensatory mechanisms that include increased activity of the sympathetic nervous system. The *middle* stage is one in which these compensatory mechanisms are no longer effective at maintaining a homeostatic cardiovascular state. This middle stage exhibits reduced cardiac output, and redistribution of blood flow to essential organs (heart and brain) in preference to skin, splanchnic organs, and kidneys. It is in this stage that many of the clinical features of

shock will be evident (i.e. oliguria, tachycardia, and tachypnea). In the *late* stage there is pooling of blood peripherally, reduced central venous pressure, intravascular fluid and protein extravasation, inadequate coronary perfusion, acute heart failure, and severe hypotension. The shock-induced reduction in intravascular volume and decrease in blood pressure are treated clinically (with varying degrees of success) by the use of intravenous fluid infusion (96,118,178) and pharmacotherapy (15,31,161,268).

1.4.1 Endotoxemia and septic shock

Septic shock (Fig. 6) is the third most frequent form of shock syndrome. There are over 100,000 deaths per year in the United States of America alone (approximately 4 deaths per 10,000 of the total population) that are attributed to septic shock, and the prevalence of this condition appears to be increasing despite modern treatment strategies (2,35). Septic shock is a clinical syndrome that has been traditionally recognized as being the consequence of gram-negative bacteremia, although it can also be caused by gram-positive bacteremia, fungi, parasitic and viral infections. Referred to in the past as endogenous suicide (189,190), septic shock has been plagued with confusion concerning its definition (2,182). What exactly is septic shock and how is it different from sepsis or mere infection? The American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference (1992) presented definitions for sepsis and septic shock and defined the latter as: "*sepsis with hypotension, despite adequate fluid resuscitation, along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status*....." (167).

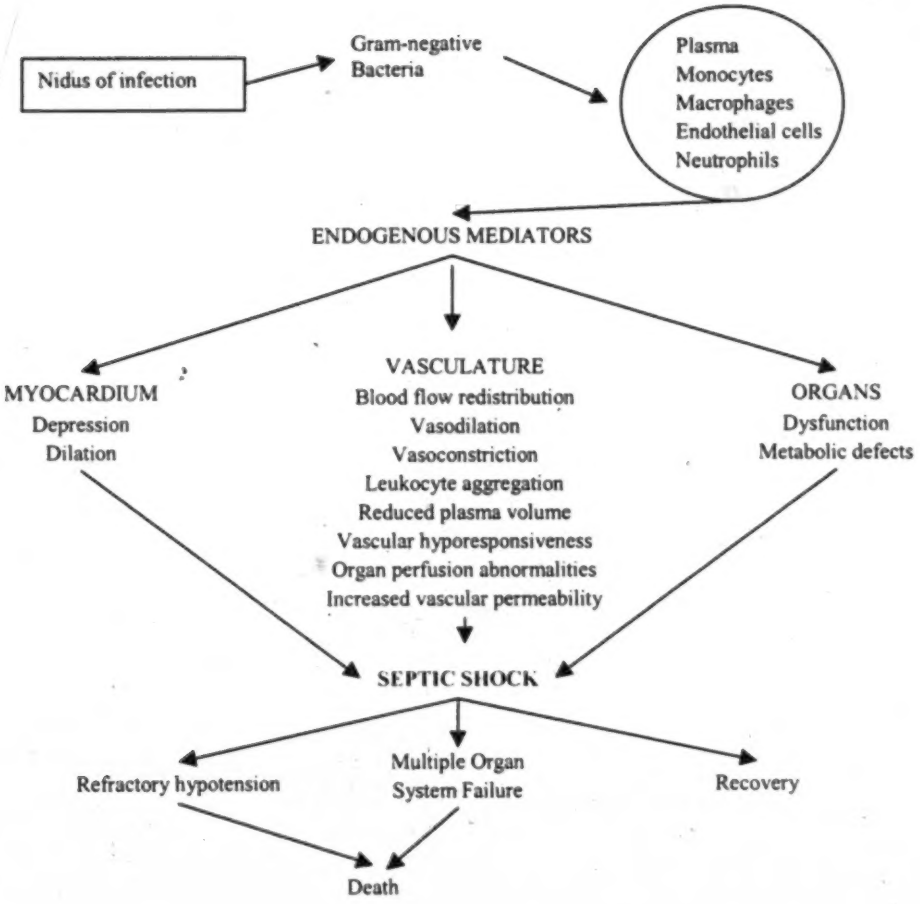

Figure 6. Characteristic pathophysiological features underlying septic shock. Illustration adapted from Parrillo (1993) (190).

Sepsis itself is a systemic inflammatory response to an active infectious process in the host, whereas septic shock is a subset of severe sepsis and is defined as a sepsis-induced hypotension (167). The clinical manifestations of septic shock include fever or hypothermia, tachypnea, tachycardia, leukocytosis, altered organ system function or perfusion, and coagulation abnormalities. The pathogenesis of septic shock progresses through four stages, namely, infection, toxin production, generation of inflammatory mediators, and finally the associated hemodynamic changes (14,196). Injection of endotoxin (which is the toxic component of the LPS cell wall of Gram-negative bacteria) to induce endotoxemia has been used to reproduce the characteristic pathophysiological features of gram-negative bacteremia and subsequent activation of the host systemic inflammatory response (257). The chemical mediators that are released by the host in response to endotoxin have global effects on the cardiovascular system (189) and cause major alterations at the microcirculatory level (162).

1.4.2 The cardiovascular system in septic shock

The changes in hemodynamic response over the course of septic shock reflect a balance between two events, namely the actions of host cardiovascular mechanisms that attempt to maintain MAP and an overwhelming systemic inflammatory response that causes hemodynamic perturbations. Sepsis in both humans and rats may exhibit a hyperdynamic or a hypodynamic circulatory response (85,216,217,244). A hyperdynamic response, which typically arises first, is characterized by an elevated cardiac output, increased tissue perfusion, hypermetabolism and decreased TPR. A hypodynamic response is

characterized by a depression in microvascular perfusion, reduced cardiac output, and hypotension (244).

Progression through the hyperdynamic and hypodynamic stages of septic shock can be altered by therapeutic intervention (196). Body compositional measurements in human hospital patients in the late stage of septic shock, reveal an expanded total-body water and interstitial water coupled with a contracted plasma volume and intracellular fluid (128,154). Such findings are typically a consequence of therapeutic intervention, whereby massive infusions of crystalloids has occurred; these expand the interstitial fluid compartment and only minimally correct plasma volume deficits (118). Indeed, hypovolemia may be present in the face of massive peripheral edema (129), which may cause further tissue damage by increasing the diffusion distance and time for oxygen to travel from the intravascular space to tissue cells (168,169).

A biphasic cardiovascular response to endotoxin has been reported in the rat (85) and also in human studies (216,217,253). This biphasic response is characterized by a rapid hypotension with partial recovery, followed shortly by a secondary hypotensive state associated with hyporesponsiveness to vasoconstrictors (234). Gardiner et al (1994) has described the cardiac hemodynamic effects of a chronic non-lethal, low dose i.v. infusion of LPS (E. coli serotype 0111:B4; 150μg/kg/hr) for 24 hr in conscious rats (88). After 1 hr, there were no significant changes in heart rate, although there was a significant decline in cardiac index and in total peripheral conductance (88). Later, there was a significant hypotension accompanied by elevations in heart rate, cardiac index, and total

peripheral conductance (88). With this model of endotoxemia, depression of cardiac function was not considered to contribute to the hypotension (88), although myocardial depression (and a subsequent hypodynamic circulation) has been suggested to contribute to sepsis-induced hypotension (143).

Septic shock is associated with dysfunction in the circulatory system (Figs. 6 and 7) that is due to the pump (myocardium), the distribution network (vasculature), and the fluid (blood) that is circulated (143,189). The cause of myocardial dysfunction has been attributed to host production of a circulating myocardial depressant substance, which causes excessive NO production in cardiomyocytes (36,143). Vascular hyporesponsiveness to vasoconstrictor agents in septic shock has been attributed to increased vascular production of NO (245) and inactivation of catecholamines (156). Abnormalities in coagulation and fibrinolytic pathways contribute to the altered rheology (108,235). Excessive loss of plasma volume due to capillary leakage is another prominent feature of septic shock (138,241,242). Each of these abnormalities in the cardiovascular system is not immediate, but occur over a period of time from the initial pathogenic insult (14,30,196).

1.4.3 The splanchnic region during endotoxemia

The splanchnic circulation (Fig. 2), which contains such notable organs as the spleen, liver and gastrointestinal tract, participates in the regulation of circulating blood volume and systemic blood pressure (204). The splanchnic circulation is regulated by intrinsic and extrinsic mechanisms (97,207,231). During pathogenic insult, the splanchnic region

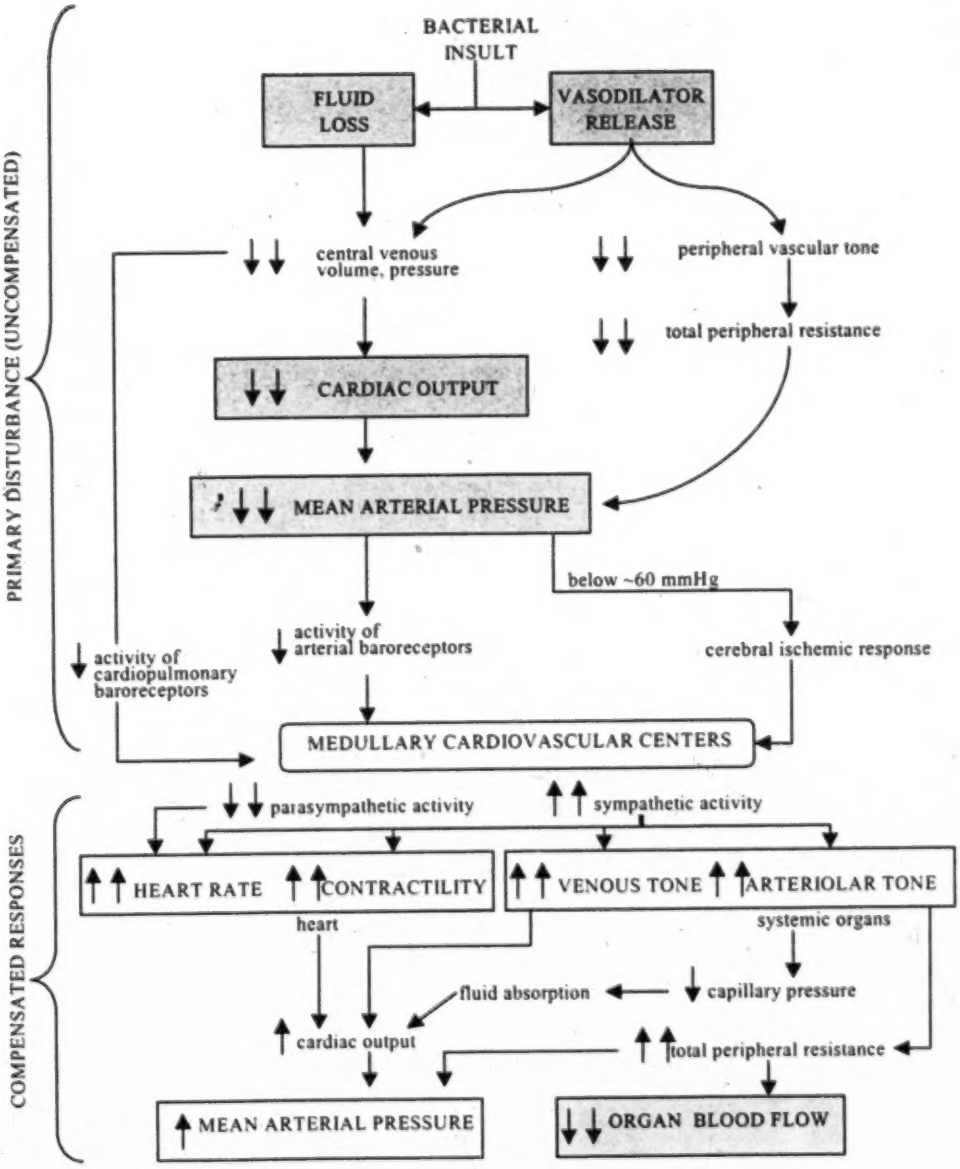

Figure 7. A brief overview of the compensatory mechanisms involved in the pathophysiology of septic shock. Reproduced from Mohrman and Heller (1997) (174).

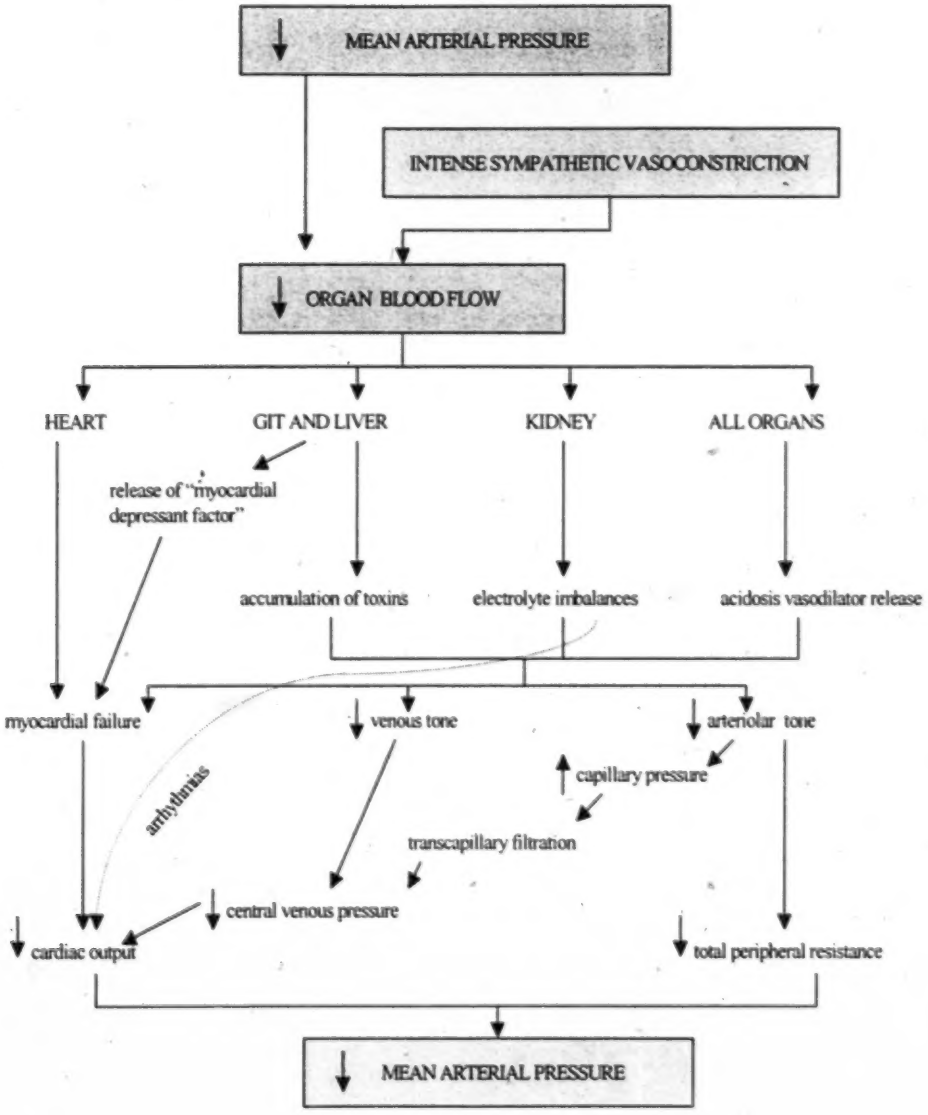

Figure 8. A brief overview of the decompensatory mechanisms involved in the pathophysiology of septic shock. Reproduced from Mohrman and Heller (1997) (174).

is an important source and target of inflammatory mediators, which may influence regional blood flow and tissue function (106,209).

There are reported changes in the distribution of regional blood flow during septicemia and endotoxemia (87,174,191,231). It has long been presumed that the reduction in splanchnic blood flow is a necessary defense during hypovolemia so as to preserve perfusion of vital organs such as the heart and brain (174). However, the increase in venous capacitance and venous pooling of blood volume within the splanchnic circulation during septic shock does impede these cardiovascular compensatory responses (14,51). Although total splanchnic blood flow is generally reduced over the course of endotoxemia (231), blood flow to the spleen actually increases during endotoxemia (7,203).

1.4.4 Microcirculatory changes during septic shock

The microvascular system consists of arterioles, venules, capillaries, sinusoids, and shunt vessels. The structure of the microvascular system reflects its functions that include, the circulation of blood within tissues and the provision of a semipermeable membrane for the exchange of substances between blood and cells of an organ. Normally, the permeability of the microcirculation is such that neither tissue edema nor intravascular hemoconcentration arises. The bacterial invasion of the bloodstream perturbs this balance (162).

At the microcirculatory level, the systemic inflammatory response is characterized by activation of the endothelium (54,115). The stimulation of these cells causes notable alterations, namely, a change in their normal anticoagulant state to a procoagulant state with increased adhesion of leukocytes and platelets to blood vessel walls, and increased release of vasoactive factors (104). During septic shock there are changes in the tone of resistance vessels throughout the body (30). The progression of microvascular changes during endotoxemia has been suggested to involve an intense arteriolar vasoconstriction followed quickly by an extreme vasodilatation with a dramatic impediment of blood flow to particular organs (162). Ultimately, the septic shock *cascade* of events brings about a state of hypotension, impaired tissue perfusion, reduced O_2, and organ failure.

Capillary leakage is a prominent feature in the later stages of septic shock, and is evident from the widespread peripheral edema, hemoconcentration, hypotension, oliguria, and tissue perfusion abnormalities (52,113,241,242). The continuous endothelium that lines most of the circulatory system, is damaged over the course of events during endotoxemia (64,198), and this enables protein-rich fluid to leak from the intravascular compartment into extravascular spaces (52,241). The poor ability of transfusions and intravenous fluid infusions to maintain vascular pressures, flow and circulatory function is indicative of a generalized increase in capillary permeability (161). The alterations in body-water distribution during septic shock may be attributed to altered Starling forces, cardiac depression, hypoalbuminemia, generalized hypotension, poor tissue perfusion, abnormal lymphatic activity and capillary leakage (108,162,192).

Despite increased capillary permeability accounting for the loss of intravascular fluid to extravascular spaces predominantly during the late stages of septic shock, it should be recognized that this feature does not influence fluid movement across the splenic microcirculation (46,135). Given the discontinuous endothelial lining of the splenic vascular bed (232), it is possible for cell-free fluid to readily cross from the intravascular space into the extravascular compartment at all times, and the extent of this extravasation depends on hemodynamic forces within the splenic circulation (46,135). Hence, during septic shock it is altered intrasplenic hemodynamics that will determine intrasplenic fluid efflux and not changes in capillary permeability. Moreover, these alterations in intrasplenic hemodynamics can increase loss of protein-rich fluid long before there is generalized vascular endothelial damage during endotoxemia.

1.4.5 The lymphatic system and response to endotoxemia

The lymphatic system has functional importance in immunity and homeostasis of fluid balance (119). It differs from the circulatory system in that it is not a closed circuit, but begins blindly in the intercellular spaces of the soft tissues of the body. The valves that occur within lymphatic vessels make it a one-way drainage system. The lymphatic network that exists in most organs has a superficial and a deep capillary system. Although it was considered at one time that there were no deep lymphatics in the rat spleen (160,267), there is now ample evidence that such drainage vessels exist within the spleen (135,142,193,211). Lymphatic fluid from the splanchnic and other vascular beds generally flow through a series of ducts, before eventually reaching the thoracic duct (119,129,265). In contrast, a large proportion of fluid that flows from the splenic

lymphatics drains into the hepatic portal vein (125). Indeed, if the hepatic portal vein is occluded, lymphatic fluid has been observed to back up into the splenic vascular arcade (Chen and Kaufman, unpublished observation).

Under normal circumstances, the continuous leakage of macromolecules (like albumin) from the plasma to the tissues is counterbalanced by a return of protein-rich fluid into the intravascular compartment from the lymphatic system (119). In humans the volume of lymphatic return to the general circulation, over a 24 hr period, is comparable to the volume of the intravascular pool (129). Approximately 50% of total circulating blood proteins that leak out of the capillaries into the tissue fluid over the course of a day are returned to the blood via the lymphatic system (129). Consequently, the lymphatic system is vital for maintaining the integrity of the circulation (119,129).

During endotoxemia, increased endothelial cell permeability of blood vessels results in greater shifts of plasma water and proteins from the intravascular compartment into extravascular spaces (241). Normally, the response of the lymphatic vessels to extravasation of fluid would be to accommodate the increased volume of lymph and to facilitate transfer of this fluid back into the intravascular compartment through increased contractile activity (128). However, the normal pumping activity of lymph vessels, which is controlled by local physiological forces (such as NO) together with inputs from neurogenic and humoral factors (ANF and angiotensin II) (69,128,215), is suppressed in endotoxemia (70,94,111). Indeed, it appears that, although endotoxin induces a rise in capillary permeability and consequently causes an elevation in lymphatic flow rates

(129), endotoxin impedes lymphatic pump activity (70,94,111). It stands to reason that the increased loss of protein-rich fluid from damaged capillaries into extravascular spaces, accompanied by a reduced return of this extravasated fluid from the lymphatic system back into the intravascular compartment due to suppression of lymphatic pumping activity, must contribute to the perturbations in plasma volume and blood pressure during endotoxemia.

1.4.6 Changes in plasma volume during septic shock

Fluid extravasation from the capillaries is enhanced by arteriolar vasodilatation and is reduced by vasoconstriction. Consequently, plasma volume should be considered, not as a static quantity but as, a fluid volume in dynamic equilibrium across the capillary wall. Hypovolemia is a major factor that contributes to the hemodynamic instability during septic shock. Several notable factors may contribute to the hemodynamic perturbations in plasma volume over the course of septic shock: i) decreased venous tone increases venous capacity, leading to venous pooling and lowered effective venous return, and ii) increased microvascular permeability and third spacing of fluid (14,239).

Animal studies have typically assumed that the reduction in plasma volume in response to endotoxin (49,51,241) is due to a generalized increase in capillary permeability, which leads to a subsequent efflux of iso-oncotic fluid into extravascular spaces (99,113). However, closer analysis of the data indicates that the early hemoconcentration and hypotension following intravenous endotoxin infusion precedes the generalized increase in capillary permeability (49-52). Furthermore, the LPS-induced hypotension and

hemoconcentration was reportedly difficult to initiate in splenectomized dogs compared to intact dogs (51). A reason for this observation was never proposed. However, given the suggested role of the spleen in the regulation of plasma volume (46,134,135) intrasplenic fluid efflux may have a role in the early hemodynamic perturbations in plasma volume and blood pressure during endotoxemia. As previously mentioned, fluid extravasation from the splenic circulation is dependent on intrasplenic P_C , which itself is determined by the vasoreactivity of splenic resistance arteries versus veins.

1.4.7 Changes in vasoreactivity of resistance vessels in septic shock

During septic shock, there is a global loss in cardiovascular control with changes in vasoconstriction and vasodilatation of regional vascular beds, the end result being a decline in TPR (108,189,191). Although the vasoreactivity of the microvasculature to circulating and locally produced vasoconstrictor and vasodilator agents is generally reduced during endotoxemia (30,115,262), changes in vascular reactivity vary between vascular beds within the microcirculation (162). There is evidence that exposure of blood vessels to LPS produces an alteration in the percent tension change generated in both conduit (224,230) and resistance (173) vessels in response to various vasoactive agents. It is believed that hyperproduction of NO by the inducible form of NO synthase (iNOS), an enzyme that is activated during endotoxemia, contributes to the hyporeactivity of the vasculature (139,262).

1.5 LIPOPOLYSACCHARIDE

LPS is situated in the outer part of the cell wall of gram-negative bacteria. LPS is a structurally complex molecule made up of highly variable and highly conserved structural regions. It consists of three components, an `O` side-chain, an inner core region, and lipid A. It is the lipid A component which is essentially `toxic` (and hence is denoted as endotoxin) (197), whereas the carbohydrate side-chain component is specific to the species of bacteria. The terms LPS and endotoxin are not strictly synonomous (80), but these two terms are used interchangeably.

1.5.1 Host recognition of lipopolysaccharide

Lewis Thomas (1974) described the recognition response between bacteria and host as : " ..... it is the information carried by the bacteria that we cannot abide. The gram-negative bacteria are the best examples of this. They display lipopolysaccharide endotoxin in their walls, and these macromolecules are read by our tissues as the very worst of bad news. When we sense lipopolysaccharide, we are likely to turn on every defense at our disposal..." (122).

There are numerous recognition molecules that exist within the host that bind LPS (11,240). A host serum protein, known as LPS-binding protein (LBP), attaches to LPS due to its ability to bind with particular molecular patterns on the bacterial molecule (213). This complex of LBP and LPS serves to mediate the attachment of LPS to the CD14 receptor on host immune cells. CD14 has been suggested to be the major host receptor for bacterial endotoxin (11) and is found on the plasma membrane of most host immune cells and in soluble form in the serum. CD14 alone can bind to LPS, but the

presence of serum LBP increases their interaction 100-1000 times (213). On binding with LPS, membrane-bound CD14 initiates intracellular signaling events within the host cell that culminates in the activation of host defense mechanisms involving both immune and non-immune cell types. Soluble CD14 is involved in the LPS-mediated activation of CD14-negative cells such as endothelial, epithelial and vascular smooth muscle cells (VSMCs) (151,195). As the spleen is the major lymphoid organ, and comprises a large number of immune cells, it can be reasoned that the spleen would be a prominent site for host recognition and defense mechanisms for LPS.

1.5.2 Host response to lipopolysaccharide following recognition

The host response to endotoxin involves the production of a variety of chemical mediators. These mediators are produced by stimulated immune (macrophages and lymphocytes) and non-immune (VSMCs and endothelium) cells (151,195). Essentially, the recognition of LPS triggers the activation of inducible genes that encode various protein mediators that include cytokines (57). These mediators up-regulate host defense mechanisms. These chemical mediators act typically in cascade pathways (which can be linked to one another or independent) (68).

1.5.3 Cytokines: the initial host molecules released in response to lipopolysaccharide

Cytokines are a group of proteins that are synthesized and released early in the host response, and are considered to mediate many of the effects of endotoxin (105). The synthesis of cytokines is controlled at the level of gene transcription (105). Cytokines are

released from a variety of cell types including immune, endothelial and VSMCs (29,105). The circulating levels of pro-inflammatory cytokines increase rapidly after pathogenic insult in human and rat models of septic shock (105,247). Excessive release of pro-inflammatory cytokines into the circulation contributes to the overwhelming systemic inflammation that arises with pathogenic invasion of the bloodstream.

Tumour necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-β), interleukin-6 (IL-6), and interleukin-8 (IL-8), the so-called pro-inflammatory cytokines, have been considered as the cytokines most prominently involved in the early events of septic shock (29,105). A major source of cytokines is the macrophage (29), and the spleen houses a large quantity of this type of immune cell. However, in the absence of the cytokine production from sources such as the spleen (as a consequence of splenectomy) (221), other tissues are capable of producing significant amounts of these proteins (29). Indeed, endotoxin has been shown to stimulate the production of the key cytokines IL-1, TNF and IL-6 in both cultured VSMCs (250) and in an isolated rat aortic ring preparation (163). This suggests that *in vivo*, blood vessels are one of perhaps many tissues that may contribute to an endotoxin-induced elevation in plasma cytokines (105).

1.6 MEDIATORS OF LIPOPOLYSACCHARIDE-INDUCED HEMODYNAMIC PERTURBATIONS

ADM, NO, endothelin-1 (ET-1) and ANF plasma levels are elevated during septic shock (5,114,139,179,183,227,245,254). These vasoactive agents may play a role in the hemodynamic perturbations that occur in endotoxemia. Specifically, each of these

vasoactive agents may affect splenic hemodynamics in a manner that elevates intrasplenic P_C and causes increased iso-oncotic fluid extravasation from the splenic circulation. These mediators will be discussed below.

1.6.1 Adrenomedullin

ADM is a vasodilator peptide (140) that is produced and secreted by VSMCs (226) and endothelial cells (225). Physical factors such as volume overload and rapid ventricular pacing can activate ADM gene transcription (131), but the most well characterized activators of ADM production are LPS and the cytokines (227). Binding site analysis in the rat has indicated dense populations of ADM receptors within the spleen and other tissues (188). ADM receptors and associated signal transduction pathways leading to vasorelaxation of VSMCs have been reviewed (170) (Fig. 9), as have the known natriuretic and diuretic actions of this peptide (210).

Plasma concentrations of ADM have been reported to increase approximately 45-fold in patients with septic shock (114,183). Intravenous infusion of LPS has also been reported to induce an increase in plasma ADM levels and in tissue ADM mRNA levels in the rat (218). The LPS-induced mediators, TNF and IL-1, increase production of ADM (227). The time-course of the increase in plasma ADM levels during endotoxemia in the rat, induced either by cecal ligation and puncture (CLP) or intravenous infusion of LPS, has been reported (170,248). The results suggest that the levels of ADM rise quickly (within a few hours) in response to a pathogenic insult.

The reduced vascular tone that characterizes septic shock has been suggested to be partly a consequence of increased levels of circulating vasodilators such as ADM (183). ADM could also affect intravascular volume by causing excessive arteriolar vasodilation, which would promote the extravasation of fluid from capillaries into the interstitium (Fig. 5). Thus, ADM may contribute to the early LPS-induced fall in plasma volume and MAP via its effects on vascular tone and fluid exchange in capillary beds. Moreover, the vasodilatory actions of ADM on splenic resistance vessels could specifically affect splenic pre- versus post-capillary tone and thus the intrasplenic P_C , thus driving iso-oncotic fluid shifts within the splenic circulation.

1.6.2 Atrial natriuretic factor

ANF is a peptide involved in cardiovascular homeostatic mechanisms through its actions on the vasculature, kidneys, and brain (146). Although ANF is typically considered as a vasodilator, there is evidence that ANF has vasoconstrictor actions on the splanchnic and coronary vasculature (228,259-261). ANF and the receptors for this peptide have been found in the spleen (144,236). Evidence from an anesthetized blood-perfused spleen preparation in the rat shows that direct infusion of ANF into the spleen predominantly causes post-capillary vasoconstriction (228). The A-type natriuretic peptide receptor has been suggested to mediate this ANF-induced vasoconstriction (228), perhaps uncharacteristically via a pathway involving cGMP production (246) or by provoking the secondary release of an autocrine- or paracrine-released vasoconstrictor such as ET-1 (71) (Fig. 10).

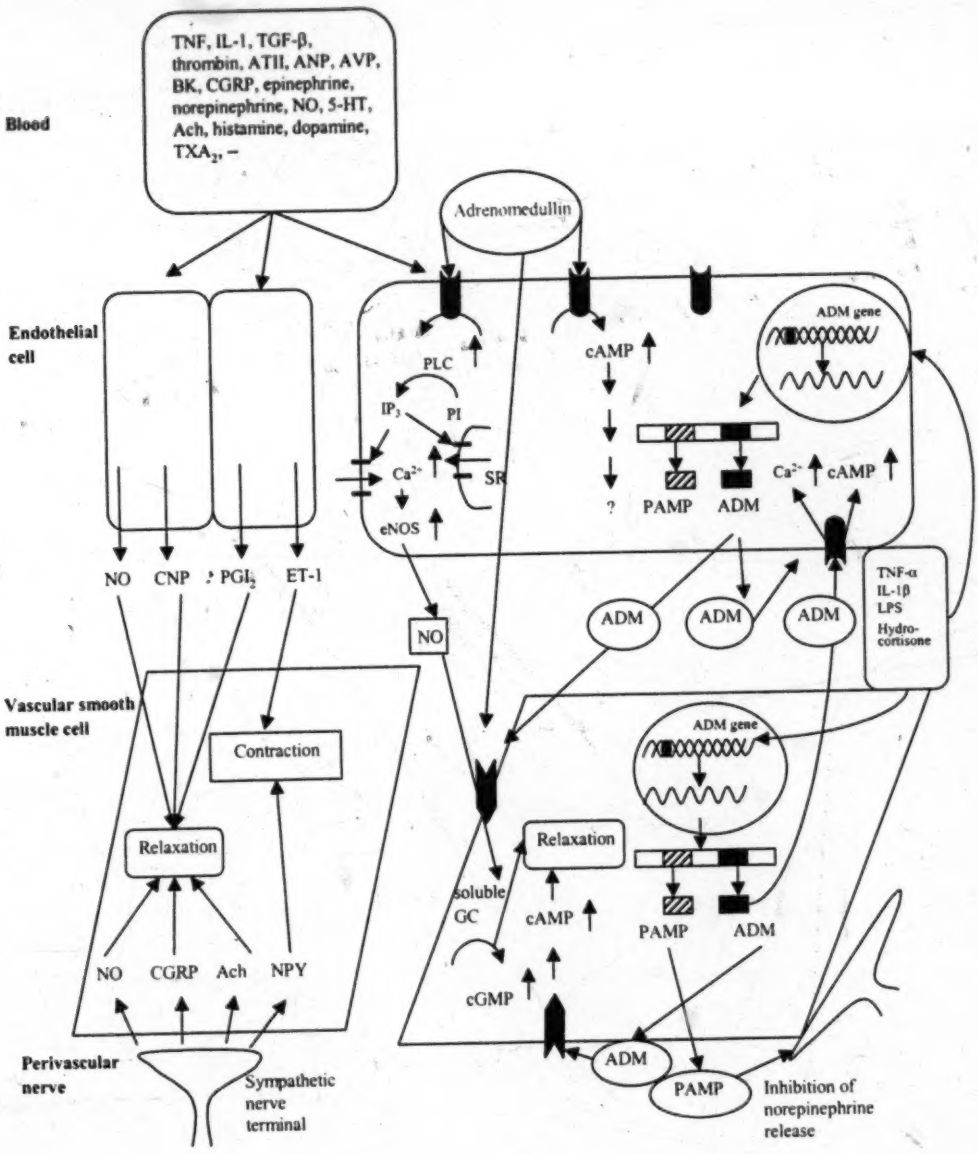

Figure 9. Adrenomedullin (ADM) in the vascular wall and mechanisms of vasorelaxation. Reproduced from Minamino et al (1998) (170).

Exogenous ANF administration causes hemoconcentration and hypovolemia (6), which cannot be accounted for by urinary excretion (61). Indeed, these early ANF-induced effects are significantly attenuated by splenectomy (134). Hence, a possible link between ANF-induced reduction in intravascular volume and intrasplenic fluid extravasation has been proposed (134). Intrasplenic infusion of ANF into anesthetized rats causes a dose-dependent increase in splenic venous hematocrit in comparison to the arterial hematocrit (66) i.e. there is loss of cell-free fluid within the splenic circulation. Moreover, in response to volume loading or atrial distention (both stimulants for ANF release) there was also a relative increase in the hematocrit of blood draining from the splenic circulation (135).

Direct infusion of ANF into a blood-perfused spleen of a rat has been shown to cause a dose-dependent increase in intrasplenic P_C (228). Altogether, the major conclusion from these studies is that the spleen has an important role in the reduction in plasma volume caused by physiological doses of ANF, due to enhanced intrasplenic P_C and subsequent shift of iso-oncotic fluid from the intravascular compartment into the systemic lymphatic system.

The time-course and amount of ANF release are important with respect to the link between septic shock, plasma ANF concentration, and regulation of intravascular volume. Circulating concentrations of endogenous ANF rise in response to intravenous endotoxin infusion in the rat (5). The rise in circulating ANF levels occurs within hours of the administration of a bolus dose of LPS (5). Hence, it is plausible to propose that

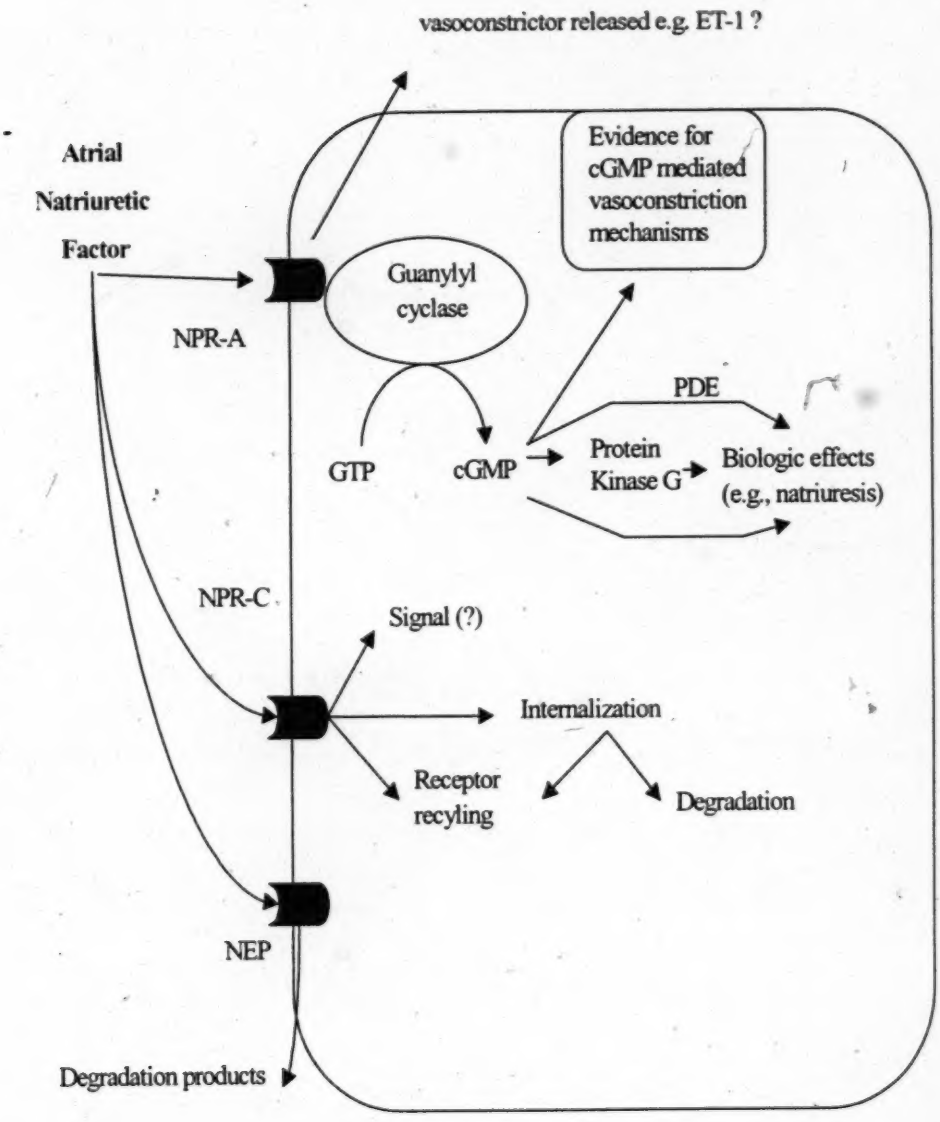

Figure 10. Possible cellular mechanisms of atrial natriuretic factor (ANF) to produce vasoconstrictor effect. Illustration adapted from Levin et al (1998) (146).

increased ANF levels during endotoxemia may contribute to the intrasplenic hemodynamic changes that promote fluid shift from the splenic circulation.

1.6.3 Endothelin-1

ET-1 is a potent vasoconstrictor peptide (266) whose synthesis is enhanced by numerous stimuli, including LPS and the cytokines (205). The main regulatory point determining the bioavailability of this peptide is the transcription of the ET-1 gene (44). The vascular endothelial cell, which constitutively releases ET-1, appears to be the major source of this vasoactive peptide (44). Two receptor subtypes, designated ET_A and ET_B, mediate the vasoconstrictor effects of ET-1 on VSMCs, but ET_B receptors situated on endothelial cells mediate ET-1-induced vasorelaxation (44) (Fig. 11). ET-1-induced vasoconstriction is long lasting and occurs in both arteries and veins. However, there is evidence that veins are more sensitive to ET-1-induced vasoconstriction than arteries in mesenteric (62) and pulmonary (208) vessels.

The plasma concentration of ET-1 is elevated in septic patients (254) and in rats administered LPS intravenously (179). Hence, the LPS-induced increase in ET-1 levels may contribute to the hemodynamic perturbations that arise during endotoxemia. In terms of the splenic circulation during infusion of LPS into the bloodstream, ET-1 may contribute to the vascular tone developed by pre- versus post-capillary splenic resistance vessels. If ET-1 was a more potent vasoconstrictor of splenic resistance veins than arteries, as appears the case in other parts of the microcirculation (62,208), then this vasoactive peptide may be proposed to contribute to a rise in intrasplenic P_C.

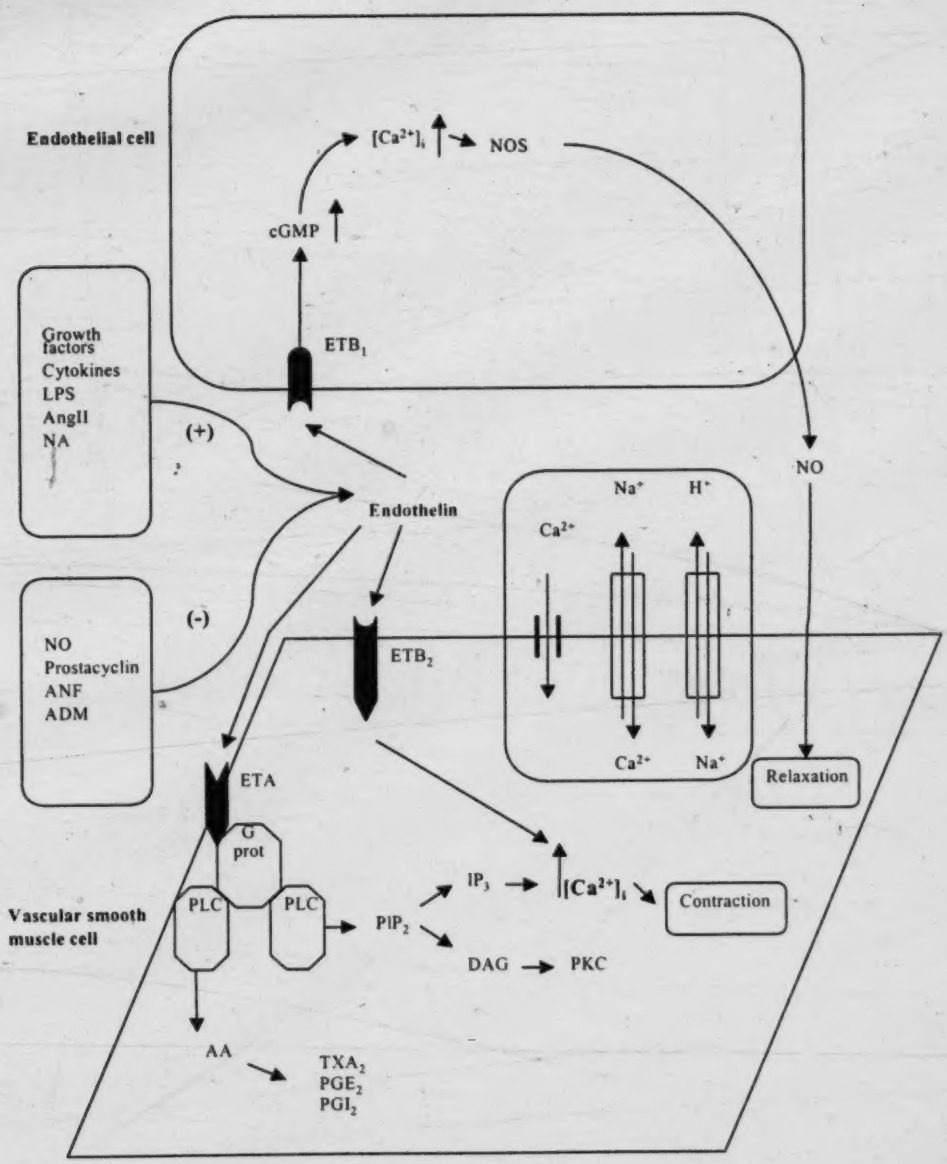

Figure 11. Cellular mechanisms of endothelin-1 (ET-1) mediated effects on vascular tone. Reproduced from Chabrier (1993) (44).

1.6.4 Nitric oxide

NO plays a primary role in determining vascular tone of resistance vessels (98). NO is a short-acting, highly labile molecule synthesized by endothelial cells (84) and by VSMCs (45) through the activity of the enzyme, nitric oxide synthase (NOS). NOS enzymes exist in three isoforms, designated as NOS I (or neural constitutive NOS or ncNOS), NOS II (or inducible NOS or iNOS), and NOS III (or endothelial constitutive NOS or ecNOS) (83,141). Shear stress is a major physical stimulant of ecNOS-derived NO from the endothelium, and LPS is a major stimulant of iNOS-derived NO in the cardiovascular system (83). Activation of iNOS results in an uncontrolled production of large quantities of NO, unlike the more limited release of NO derived from ecNOS or ncNOS isoforms. Hormones such as ADM exert their vasorelaxant actions, at least partly, through NO generation (170) (Fig. 9). The routes for NO-induced vasorelaxation *in vivo* and *in vitro* are by direct action on VSMCs (55) (Fig. 12), or through inhibition of sympathetic nerve-induced vasoconstriction *in vivo* (269). Hence, the basal release of endothelial NO provides a vasorelaxant effect that opposes sympathetic vasoconstriction. Furthermore, NO has been reported to antagonize the actions of the endothelium-derived vasoconstrictor, ET-1 (92).

NO formation, which increases in human septic shock and endotoxemia in animals (139,245), serves a dual role for the host following pathogenic invasion: i) it has anti-microbial effects (181) but, ii) when overproduced, it can cause circulatory abnormalities (153). Although the role of NO in sepsis is still an area of debate (139,245), NO is

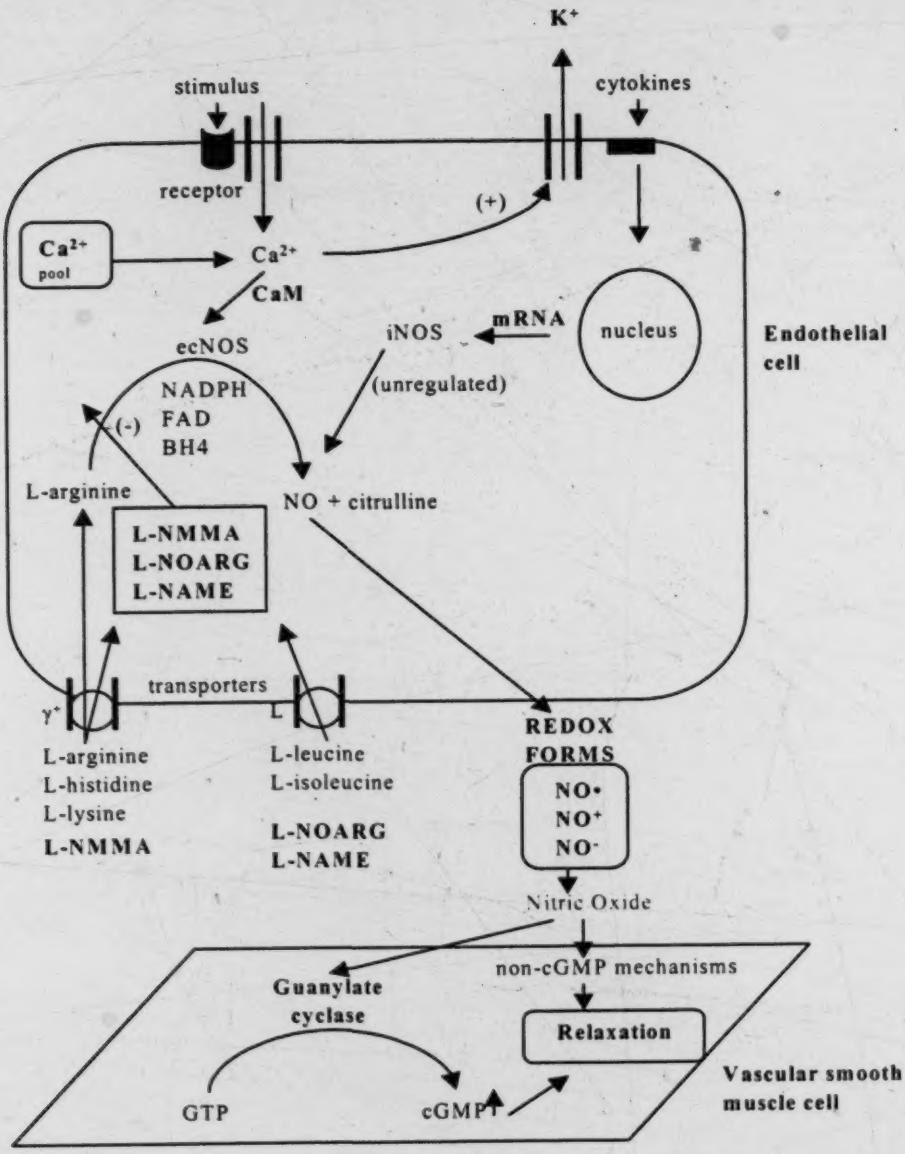

Figure 12. Mechansims of nitric oxide (NO)-induced vasorelaxation. Reproduced from Cocks (1996) (55).

generally considered as one of the major mediators involved in the cardiovascular pathophysiology of septic shock and endotoxemia (139,192,245). The rapid time course of the LPS-induced elevation of iNOS expression (149) and subsequent excessive NO production implicates this vasoactive agent in the early hemodynamic perturbations that arise in endotoxemia. Consequently, inhibition of NO biosynthesis has been investigated as a potential strategy for the treatment of septic shock (139,245).

LPS increases the expression of iNOS in numerous tissues of the rat, including the spleen (148,171). Specifically, an early LPS-induced increase in NO within the splenic circulation may contribute to the augmentation of the hemodynamics within this vascular bed. Indeed, close infusion of NO into the splenic artery increases intrasplenic P_C and fluid efflux from the splenic circulation of an anesthetized rat (8). Thus, the mechanism through which NO may alter pre- versus post-capillary tone of splenic resistance vessels *in vivo* to increase intrasplenic P_C , could be either through NO-induced inhibition of SSNA-induced pre-capillary vasoconstrictor tone and/or through differential vasoreactivity of pre- and post-capillary resistance vessels to NO-induced vasorelaxation. The former is supported by evidence that NO blunts the increased SSNA typically observed in rats after endotoxin infusion (95). The latter was addressed in this thesis by assessing the vasoreactivity of isolated splenic resistance arteries and veins to NO.

1.7 A RAT MODEL OF SEPTIC SHOCK

Developing an animal model of septic shock that is clinically relevant is fraught with complications (80,257). Essentially four main approaches have been used to initiate

features of septic shock in animal models (257): (1) the intravenous infusion of live bacteria, (2) the administration of fecal material or live organisms into the peritoneal cavity, (3) the placement of infected foreign material into soft tissues of the body in an attempt to produce abcesses, and (4) surgical operations that partially destroy the normal barriers of the gastrointestinal tract. Each of these approaches has drawbacks and each simulates only part of the hemodynamic and metabolic alterations that arise during septic shock in humans (80,85).

Host responses to endotoxin, the infusion of which is widely used to simulate gram-negative sepsis, are affected by such factors as the dose, route of administration, diurnal rhythm, interspecies differences in sensitivity to endotoxin, and light/dark cycles (38,198), although the systemic response of the host does not appear to be affected by the type of invading pathogen (258). Differences in response to endotoxin between species plus the variability caused by the aforementioned factors, has frustrated the development of a clinically relevant animal model of septic shock.

The basic response to sepsis (namely hypotension, increased cardiac index, and reduced vascular resistance) that arises in humans is also found in rat models (80,257). We chose to use a continuous low dose infusion of LPS (E. coli serotype 055:B5; 150μg/kg/hr) in conscious unrestrained male rats, because we were interested in the early phase of clinical septicemia, where there is an insidious and progressive onset of cardiovascular anomalies. Indeed, this model of low dose infusion of LPS into rats has been reported to produce hemodynamic features similar to the early stages of septic shock in humans

(namely a hyperdynamic response) (88). Many rat model studies of septic shock have used high dose bolus infusions of LPS that have evoked high mortality rates and restricted observation times, and the clinical relevance of such an experimental approach has been criticized (257). A single bolus dose infusion of LPS is not representative of the human condition where a patient suffering from septic shock is exposed to a continuous slow leakage of endotoxin into the bloodstream (80,257). Although CLP is arguably the model of sepsis that most closely resembles the clinical development of septic shock, where there is typically a nidus of infection (257), there are significant differences between CLP and intravenous infusion of endotoxin (257). There are differences with respect to the evoked release of secondary mediators released by the host and the plasma levels of endotoxin that have been measured in each model (42,72,155,243). In brief, the studies that have compared these two models have reported that the expression of IL-1 and TNF is dissimilar (72), and the plasma levels of LPS achieved 1 hr after an intraperitoneal bolus injection of endotoxin was 40-fold higher than those found 2 hr after CLP (243). However, it should be noted that the bolus dose of LPS used in the Villa et al (1995) study (243) was approximately 200-fold higher than the total dose of LPS administered in 1 hr in the rat model of endotoxemia used by Gardiner et al (1994) (88).

We chose to use a rat model to examine the role of the spleen in the control of plasma volume and blood pressure during early hemodynamic perturbations induced by LPS. First, the rat spleen is functionally and anatomically similar to the human spleen (199): as the rat possesses a similar sinusoidal network and splenic microcirculatory pathways (fast and slow routes of blood flow) as do humans, and red blood cell washout experiments

have also been quantitatively similar (100,101). Second, the absence of splenic storage of blood volume and hypovolemia-induced expulsion of high hematocrit blood into the circulation in the rat, as occurs for example in the dog and horse (199), would not undermine interpretation of hemodynamic measurements during endotoxemia.

1.8 EXPERIMENTAL PREPARATIONS AND HYPOTHESES

We utilized four main experimental preparations during our investigations, which were:

1. *in vivo* experiments using conscious unrestrained rats infused with non-lethal, low dose LPS over a period of 18 hours, during which time hemodynamic measurements were taken. Comparisons were made between rats that were: i) intact versus splenectomized, and ii) intact versus splenic denervated.
2. *in vivo* experiments using anesthetized rats, in which splenic arterial and venous hematocrit were measured during intrasplenic infusion of ADM and a NO donor agent into the splenic artery.
3. a blood-perfused spleen preparation utilizing anesthetized animals, in which ADM was infused directly into the splenic artery, and the effect on intrasplenic P_C was assessed.
4. an *in vitro* wire myograph preparation, which was used to assess the vascular reactivity of splenic resistance arteries and veins (that were either normal or had been exposed to LPS for 3 hours *in vivo*) to the vasoactive agents ADM, ANF, ET-1, and NO.

The first series of *in vivo* experiments examined the effect of splenectomy on the hemodynamic perturbations following infusion of LPS. Based on evidence that the spleen was a pathway for iso-oncotic fluid efflux (46,134,135), we proposed that:

splenectomy would significantly blunt the early LPS-induced hypotension, hemoconcentration, and hypovolemia.

The second series of *in vivo* experiments investigated the effect of splenic denervation. We proposed that the LPS-induced increase in SSNA limits the rise in intrasplenic P_C. Therefore, in the absence of splenic innervation, a greater rise in intrasplenic P_C would be possible. Thus, we considered that splenic denervation should exacerbate intrasplenic fluid efflux during endotoxemia, and so exaggerate the hemodynamic perturbations following infusion of LPS. We sought to test the hypothesis that:

splenic denervation would significantly exacerbate the initial LPS-induced hypotension, hemoconcentration, and hypovolemia.

Part of the evidence for intrasplenic fluid efflux was an elevation in the hematocrit of blood leaving the spleen via the splenic vein compared to the splenic artery in response to stimuli such as hypervolemia and ANF (134,135). On the basis of these findings, we sought to measure the changes in splenic arterial and venous hematocrit following the close infusion of ADM and NO into the splenic artery of anesthetized rats. We hypothesized that:

both ADM and NO would significantly increase splenic venous hematocrit compared to splenic arterial hematocrit.

An elevation in intrasplenic P_C has been suggested to be the force that drives intrasplenic fluid efflux (228). Since ADM levels increase approximately 40-fold during septic shock (114,183), we sought to investigate whether intrasplenic infusion of ADM would elevate intrasplenic P_C. To this end, the double vascular occlusion technique was utilized in a blood-perfused spleen to determine ADM-induced changes in intrasplenic P_C (20,228,238). We hypothesized that:

ADM would significantly increase intrasplenic P_C.

As previously discussed, differential vasoreactivity of splenic resistance vessels to vasoactive agents could underlie the necessary net increase in intrasplenic P_C that drives intrasplenic fluid extravasation. *In vitro* studies of splenic resistance vessels, mounted on a wire myograph, assessed vasoreactivity to ADM, ANF, ET-1, and NO. Each of these vasoactive agents increase in both human and animal studies of septic shock, and are implicated in the hemodynamic perturbations that follow LPS infusion. Our hypothesis stated that:

there would be differential vasoreactivity between splenic resistance arteries and veins to ADM- and NO-induced vasorelaxation, and ANF- and ET-1-induced vasoconstriction.

In addition, given the characteristic development of vascular hyporesponsiveness to vasoactive agents in septic shock, we investigated whether there was maintenance of this

vascular reactivity to ADM, NO and ET-1 in 3-hr LPS-exposed splenic resistance vessels. Our hypothesis stated that:

differential vasoreactivity would be maintained between 3 hr LPS-exposed splenic resistance arteries and veins to ET-1-induced vasoconstriction, and NO- and ADM-induced vasorelaxation.

CHAPTER 2.

MATERIALS AND METHODS

The *in vivo* experiments (A, B, C and D) were examined by the local Animal Welfare Committee, University of Alberta and found to be in compliance with the guidelines issued by the Canada Council on Animal Care. At the completion of an experiment, all animals were killed with an anesthetic overdose (0.3 ml Euthanyl; intravenous, iv; 240mg/ml sodium pentobarbital; MTC Pharmaceuticals, Cambridge, Ontario, Canada).

Animal model: Male Long Evans rats (450-600g) were obtained from Charles River, St. Foy, Quebec, Canada. They were held in the University Animal Facility for at least one week prior to experimental procedures, exposed to light of 12/12 hour cycle, in a humidity and temperature controlled enviroment, and maintained on a 0.3% sodium diet and water ad libitum.

EXPERIMENT A: EFFECT OF SPLENECTOMY ON LIPOPOLYSACCHARIDE (LPS)-INDUCED HYPOTENSION, HEMOCONCENTATION AND HYPOVOLEMIA IN CONSCIOUS MALE RATS.

Surgery (Expt A): Sodium pentobarbital (62 mg/kg body wt.) was administered i.p., followed by penicillin (0.1 ml i.m. Ethacillin Rogas/STB Inc, London, Ont. Canada) and atropine (0.1 ml, 0.4 mg/ml). Buprenorphine (0.01mg/kg) was given after the completion of surgery. Throughout the surgical procedures, the rats were maintained on a Deltaphase isothermic heating pad (Braintree Scientific, Inc., Mass., USA) which maintained body temperature at ~37°C. Isotonic saline (4 ml/hr) was infused i.v. into each animal throughout the surgery.

The jugular vein was cannulated using Silastic tubing (0.51 mm ID x 0.94 mm OD). A mid-line laparotomy was then performed. Two cannula were placed non-occlusively into the inferior vena cava (Silastic 0.51 mm ID x 0.94 mm OD, and polyethylene (PE10, 0.28mm ID x 0.61mm OD) (133); these were for blood sampling and injection of the Evan's Blue dye respectively. A pressure transmitter (PA-C40, Data Sciences International, Minnesota, USA), was implanted in the abdominal aorta (mid-way between the branch to the left renal artery and the bifurcation to the femoral arteries). This latter device enabled continuous measurement of MAP. Rats were subjected to either splenectomy or sham-operated (but left intact). All animals were allowed one week to recover from surgery and to regain their preoperative body weight.

Measurement of hematocrit, plasma protein concentration and urine output (Expt A): Blood samples (50μl) were taken from the central venous catheter into heparinized microhematocrit tubes. They were centrifuged and read immediately after collection. The protein content of the plasma (which was separated from the red blood cell mass) was read immediately using a refractometer. Urine volume (over a 24 hr period) was collected in a graduated urine volume separator.

Measurement of heart rate (Expt A): The heart rate (beats/min) was calculated by analysis of the MAP data using Windaq software (Windaq, DATAQ Instruments Inc, Akron, Ohio). Heart rate was determined by counting the number of spikes in the MAP recordings from data collected for a 5 min period before each timed blood sample

collection; at 0 min, 20 min, 40 min, 60 min, 90 min, 3 hr, 8hr and 18 hr post-LPS or saline infusion.

Measurement of blood volume (Expt A): Plasma volume was determined by means of the Evan's blue dye dilution method. In short, initial blood samples (0.25 ml) were taken. A solution (0.3 ml, 0.5 g/100ml in sterile isotonic saline) of Evan's blue (Baker Chemical Co., Phillipsburg, NJ) was injected via the smaller indwelling venous cannula. The line was flushed with 0.2 ml saline. At 10, 20, 30, 40, and 60 min, blood samples (0.15 ml) were taken from the larger venous cannula, rapidly transferred to heparinized Fisherbrand Caraway tubes (Fisher Scientific, Edmonton) and centrifuged. The hematocrit was measured and the plasma was separated from the red blood cells. Meanwhile, the blood sample was replaced with the same volume of saline. The plasma samples (50 µl) were diluted in 950 µl saline and absorbance was measured at 605 µm on a Spectrophotometer (LKB Biochrom, model 4049, Cambridge, England). The readings were compared with standards obtained by adding 0, 1, 2 µl of the 0.5 % Evan's blue solution to 50 µl initial plasma plus 950 µl saline. The plasma volume and blood volume were calculated by extrapolation back to time zero.

Lipopolysaccharide (LPS) infusion (Expt A): LPS (150 µg/kg/hr), derived from Escherichia coli (serotype 055:B5), was supplied by Sigma Chemicals as a lyophilized powder, chromatographically purified by gel filtration, with a protein content of <1 %. One week after surgery to implant the indwelling cannulae, an osmotic mini-pump (Alza Corporation, Palo Alto, CA, USA) was implanted under isoflurane anesthesia (Abbott

Labs, Montreal, Canada) and connected to the jugular vein cannula. The length of the jugular cannula (80 mm) gave a "lead-in" time of exactly 2 hr at the delivery rate of 8 µl/hr i.e. time zero was 2 hr after implantation of the pump, at which time the rat was conscious and fully recovered from the brief period of anesthesia. Infusion of LPS continued over the entire 18 hr experimental period, and with a mean body weight per rat of 530 $\pm$ 7 grams the total dose of LPS over the entire 18 hr infusion period was approximately 1.43 $\pm$ 0.1 mg.

Protocol for MAP (Expt A): Basal (-24 hr) measurements of MAP were determined on the day before the start of the experiment. The start of the experimental measurements (time "0 min") was defined as the commencement of LPS (or saline) infusion into the jugular vein. MAP was continuously recorded and later analyzed (Windaq, DATAQ Instruments Inc, Akron, Ohio). MAP was derived from data collected for a 10 min period before each timed blood sampling.

Protocol for hematocrit, plasma protein concentration and urine volume (Expt A): Basal (-24 hr) measurements of hematocrit were determined on the day before the start of the experiment. Serial measurements of hematocrit and plasma protein concentration were derived from blood samples taken at 0 min, 20 min, 40 min, 60 min, 90 min, 3 hr, 8 hr, and 18 hr post-saline/LPS infusion into the jugular vein. Urine volume was collected 24 hours before the start of the experiment, and over a 24 hour period starting at time=0.

Protocol for blood volume (Expt A): Basal (-24 hr) blood volume was determined on the day before the start of the experiment, and then the following day at 90 min post-saline/LPS infusion into the jugular vein.

Experimental groups (Expt A): There were four experimental groups that were investigated in a randomized order: (i) non-splenectomized (intact) rats given LPS (n=9); (ii) non-splenectomized (intact) rats given isotonic saline (n=4); (iii) splenectomized rats given LPS (n=7); (iv) splenectomized rats given isotonic saline (n=4).

Statistical analysis (Expt A): The significance of changes in MAP, percent hematocrit, plasma volume, plasma protein concentration and urine output across time were analyzed by a one-way repeated measures ANOVA. Differences between experimental groups at each time period was analyzed using a two-way repeated measures ANOVA, followed by the Student-Newman Keuls method to identify the individual points of significance. If the data were not normally distributed, a repeated measures ANOVA on ranks was used, followed by Dunn's method to identify individual points of significance. Plasma volume was analyzed using an unpaired Student's t-test to assess the absolute decrease in plasma volume between intact and denervated LPS-infused groups. Significance was accepted at $p<0.05$.

EXPERIMENT B: EFFECT OF SPLENIC DENERVATION ON LIPOPOLYSACCHARIDE (LPS)-INDUCED HYPOTENSION, HEMOCONCENTRATION AND HYPOVOLEMIA IN CONSCIOUS MALE RATS.

Surgery and cannulations (Expt B): As described in experiment A.

Denervation of the spleen (Expt B): The splenic nerve was first visualized under a microscope and the splenic bundle (which consists of the splenic artery, splenic vein and splenic nerve) was carefully cleared from the surrounding pancreatic and connective tissue. At the cleared section of splenic bundle, the splenic nerve was detached from the splenic vessels, and a 2 mm section of nerve was cut away and discarded. The severed ends of the splenic nerve were painted with liquefied phenol solution (Fisher Scientific, Edmonton, Alberta, Canada).

Implantation of MAP recording device (Expt B): As described in experiment A.

Implantation of blood flow probes (Expt B): The use and calibration of these probes has been previously described (46). Flow probes (1RB series: Transonic Systems Inc, Ithaca, NY, USA) (Appendix A) were placed around the splenic artery and vein and the probe leads sutured securely to the body wall. Splenic blood flows were continuously recorded on-line using a flowmeter (Transonic Systems Inc, Ithaca, NY, USA) plus Windaq software (Windaq, DATAQ Instruments Inc, Akron, Ohio).

Measurement of hematocrit and blood volume (Expt B): As described in experiment A.

Measurement of plasma renin activity (PRA) (Expt B): PRA was determined in blood samples collected at time intervals of basal (- 24 hr), and then at 90 min, 8 hr, and 18 hr post-infusion of either saline or LPS. The PRA was measured using an Angiotensin I radioimmunoassay kit (Mandel Scientific, Guelph, Ontario, Canada). The subsequent reports on the accuracy, precision, and sensitivity of this assay are taken from data provided in the NEN Angiotensin I radioimmunoassay kit handbook. The accuracy of the method for the PRA assay was evaluated by determining the recovery of 0, 1, 2, 5, and 10 ng of Angiotensin I added per ml of plasma sample, and the reported percent recovery was >90 %. The intra-assay and inter-assay coefficient of variation was evaluated by analysis of replicate aliquots of four plasma pools and the precision for each of these measurements was <5 % and <6 %, respectively. Moreover, the method reports an absence of non-specific interference in the assay by plasma constituents. The sensitivity of the method, defined as the mass equivalent to twice the standard deviation of the zero binding, is approximately 40 pg/ml.

Lipopolysaccharide (LPS) infusion (Expt B): As described in experiment A.

Measurement of total catecholamine content of splenic tissue after denervation (Expt B): The total catecholamine content of intact and denervated splenic tissue (post-infusion of either saline or LPS) was measured using a catecholamine [^{3}H] radioenzymatic assay system (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). The subsequent reports on the sensitivity and precision of this assay are taken from data provided in the catecholamine [^{3}H] radioenzymatic assay system handbook. The expected sensitivity of

this assay for each individual catecholamine (noradrenaline, adrenaline and dopamine) is from 2.5 pg for noradrenaline and adrenaline and 15-20 pg for dopamine per 50 µl volume of plasma analyzed. The intra-assay coefficient of variation for noradrenaline, adrenaline and dopamine measurements was <5 %. The inter-assay coefficient of variation for each of these catecholamines was <15 %.

Measurement of wet splenic tissue weight (Expt B): Following the 18 hr infusion of saline or LPS, the rats were decapitated and the spleen removed. The wet weight of the spleen was measured.

Measurement of tumor necrosis factor-alpha (TNF-α) in plasma (Expt B): The *TNF-α* concentration was determined in plasma samples collected at time intervals of basal (- 24 hr), 90 min and 3 hr post-infusion of either saline or LPS. The *TNF-α* concentration of plasma was measured using a *TNF-α* [(r) *TNF-α*] rat ELISA system (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). The subsequent reports on the specificity, precision, sensitivity and recovery of this assay are taken from data provided in the handbook that accompanied the assay (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). The ELISA is specific for measurement of natural and recombinant rat *TNF-α* ; it does not cross-react with rat IL-1α or IL-1β. All our plasma samples were analyzed in a single assay. The intra-assay coefficient of variation of the ELISA was determined to be <10 %. The reported sensitivity and cytokine recovery of this ELISA in plasma was reported as <10 pg/ml and ≥100 %, respectively.

Measurement of heart rate (Expt B): The heart rate (beats/min) was calculated by analysis of the MAP data using Windaq software (Windaq, DATAQ Instruments Inc, Akron, Ohio). Heart rate was determined by counting the number of spikes in the MAP recordings from data collected for a 5 min period before each timed blood sample collection; at 0 min, 20 min, 40 min, 60 min, 90 min and 3 hr post-LPS or saline infusion.

***Protocol for splenic blood flow (Expt B)*:** The start of the experimental measurements (time "0 min") was defined as the commencement of LPS (or saline) infusion into the jugular vein. Splenic blood flows were derived from data collected for a 10 min period before each timed blood sample collection; at 0 min, 20 min, 40 min, 60 min, 90 min, 3 hr, 8 hr, and 18 hr post-LPS infusion into the jugular vein. Splenic arterial and venous blood flows were continuously recorded and later analyzed (Windaq, DATAQ Instruments Inc, Akron, Ohio).

***Protocol for MAP, hematocrit and blood volume (Expt B)*:** As described in experiment A.

***Protocol for TNF-α ELISA (Expt B)*:** Serial measurements of plasma TNF-α concentration were derived from blood samples taken at 0 min, 90 min and 3 hr post-saline/LPS infusion into the jugular vein.

Protocol for PRA (Expt B): Blood samples for determination of PRA were drawn 24 hours before the onset of saline/LPS infusion, and at 90 min, 8 hr, and 18 hr post-saline/LPS infusion into the jugular vein.

Experimental groups (Expt B): There were four experimental groups that were investigated in a randomized order: i) intact rats infused with isotonic saline (n=8); ii) intact rats infused with LPS (n=8); iii) splenic denervated rats infused with isotonic saline (n=7); iv) splenic denervated rats infused with LPS (n=10).

Statistical analysis (Expt B): The significance of changes in splenic arterial and venous blood flows, splenic A-V difference, MAP, heart rate, percent hematocrit, plasma volume, plasma TNF-α concentration and PRA across time were analyzed by a one-way repeated measures ANOVA. Differences between experimental groups at particular time periods were analyzed by the two-way repeated measures ANOVA, followed by the Student-Newman Keuls method to identify the individual points of significance. If the data were not normally distributed the repeated measures ANOVA on ranks was used, followed by Dunn's method to identify individual points of significance. Plasma volume was analyzed using an unpaired Student's t-test to assess the absolute decrease in plasma volume between intact and denervated LPS-infused groups. Total catecholamine content was analyzed using an unpaired Student's t-test. Differences in the splenic wet weight were analyzed using a one-way ANOVA on ranks plus Dunn's test for multiple comparisons. Significance was accepted at $p<0.05$.

EXPERIMENT C: EFFECT OF INTRASPLENIC INFUSION OF ADRENOMEDULLIN (ADM) AND S-NITROSO-N-ACETYL-D,L-PENICILLAMINE (SNAP) ON SPLENIC ARTERIAL VERSUS VENOUS HEMATOCRIT AND WET SPLENIC TISSUE WEIGHT IN ANESTHETIZED MALE RATS.

Surgery (Expt C): Anesthesia was induced with sodium pentobarbital (60 mg/kg body weight; intraperitoneal, i.p.) and maintained with Inactin (ethyl-(1-methyl-propyl)-malonyl-thio-urea, 80 mg/kg body weight; subcutaneous, s.c.). Body temperature was maintained by placing the rat on a heating pad (Deltaphase Isothermal pad, Braintree Scientific, Braintree, MA, USA). Access to the abdominal organs was through a midline laparotomy. In order to ensure that the artery and vein supplied and drained only the spleen, all accessory branches running from the splenic vessels to the pancreas, stomach and surrounding tissue were ligated (Fig. 39; Appendix D). At the end of the experiment, vascular isolation was verified by infusing dye into the splenic artery (135).

Cannulations (Expt C): The spleen was carefully cleared from its attachments to the stomach and replaced in its natural position in the abdominal cavity. The stomach was then delivered through the abdominal incision, and laid on the thorax of the rat. The gastric artery, gastric vein, splenic artery and splenic vein were cleared carefully and with minimal handling given their extreme vasoreactivity. The gastric artery was cannulated with drawn-out Tygon tubing (nominal dimensions: 0.25 mm ID, 0.5 mm OD) and the gastric vein with Silastic (Dow Corning, 0.30 mm ID, 0.64 mm OD). Polyethylene (0.58

mm ID, 0.97 mm OD) and Silastic (Dow Corning, 0.51 mm ID, 0.94 mm OD) cannulae were placed in the femoral artery and vein, respectively. The arterial cannula was used as a site for arterial blood sampling. The venous cannula was used for saline infusion.

Measurement of hematocrit (Expt C): Blood samples (50 µl) were taken from the gastric vein cannula (which represents blood flow out of the spleen) and femoral artery cannula (which represents blood composition that would flow into the spleen) into heparinized microhematocrit tubes. They were then centrifuged and read immediately after collection.

Protocol (Expt C): After cannulation of the femoral vein, saline infusion was started (3 ml/hr) and continued over the duration of the experiment. The femoral artery was then cannulated. The arterial cannula was the site for blood sampling. The preparation was allowed to stabilize for 45 min. Femoral artery and gastric vein blood samples were taken after the stabilization period for hematocrit measurements. ADM was infused for 15 min at 9 ng/ml through the splenic artery. For the SNAP experiments (which utilized separate animals), the NO donor was infused for 15 min at 0.3 µg/ml through the splenic artery. At the end of the ADM (or SNAP) infusion period, blood samples were again taken from the femoral artery and gastric vein. Control experiments received saline infusion for 15 min. The splenic vessels were then ligated, and the spleen removed and weighed.

Statistical analysis (Expt C): Arterial and venous hematocrits after saline versus ADM or SNAP infusion into the splenic artery were compared using a Student's t-test for unpaired

data. Wet splenic tissue weight after saline versus ADM or SNAP infusion was compared using a Student's t-test for unpaired data. Significance was accepted at $p<0.05$.

EXPERIMENT D: EFFECT OF ADRENOMEDULLIN (ADM) ON INTRASPLENIC MICROVASCULAR PRESSURE.

Surgery (Expt D): Anesthesia was induced with isoflurane (2.5%; IsoFlo™, Abbott Laboratories, U.S.A), and continued until the femoral vein was cannulated, at which time Somnotol (sodium pentobarbital, 65 mg/ml, i.v.; MTC Pharmaceuticals, Cambridge, Ontario, Canada) was infused (50 mg/kg). Inactin (ethyl-(1-methyl-propyl)-malonyl-thio urea; 80 mg/kg, s.c.; BYK, Germany), given at the end of surgery, maintained the rat under a surgical plane of anesthesia (no paw-pinch response) for the duration of the experiment.

In order to ensure that the splenic artery and vein supplied and drained only the spleen, all branches running from the splenic vessels to the pancreas, stomach and other surrounding tissues were ligated and divided (Fig. 39; Appendix D). Vascular isolation of the spleen using this method has been confirmed elsewhere (135). Silastic (Dow Corning, USA; 0.51 mm ID, 0.94 mm OD) and PE-50 (Intramedic, USA; 0.58 mm ID, 0.965 mm, OD) cannulae were placed in the femoral vein and artery, respectively. MAP was monitored at the femoral artery, and Somnotol was administered through the femoral vein. The venous line was also used to infuse saline (3 ml/hr) to maintain adequate hydration of the animal throughout the duration of the experiment. The right common

carotid artery was occlusively cannulated using PE-90 (0.86 mm ID, 1.27 mm OD) to provide the source of oxygenated-blood for splenic perfusion.

The spleen was carefully cleared from the stomach and replaced in its natural position in the abdominal cavity. The stomach was then placed on top of the thoracic cavity and retracted, thereby exposing the gastric artery and vein, which were used to access the splenic artery and vein. The gastric artery was cannulated with drawn out PE-50 tubing (0.58 mm ID, 0.965 mm, OD), while the gastric vein was cannulated with micro-renethane (Braintree, USA; 0.30 mm ID, 0.64 mm OD). The gastric artery cannula was connected, via a 3-way adapter, to a pressure transducer (which monitored splenic arterial perfusion pressure), and to a peristaltic pump. The venous cannula was advanced to the junction of the gastric vein and the splenic vein, and was connected to a pressure transducer (which monitored venous pressure of the blood-perfused spleen). When the surgery was completed, splenic perfusion was started. At the start of splenic perfusion, heparin (0.15 ml; 10,000 i.u./ml, i.v.) was injected. The splenic perfusion consisted of oxygenated blood taken from the carotid artery, and perfused into the splenic artery via the peristaltic perfusion pump (1.0 ml/min). Systemic blood pressure and splenic arterial and venous perfusion pressures, were monitored online using a data acquisition system (D1-400, DATAQ Instruments, Akron Ohio, USA) and recorded using DATAQ's own software (WINDAQ).

Intrasplenic microvascular pressure (Expt D): In the blood-perfused spleen, microvascular pressure (P_C) was determined using the double vascular occlusion

technique (238). After stabilization, both inflow and outflow cannulas were simultaneously occluded. Arterial pressure (P_A) and venous pressure (P_V) equilibrated rapidly to a value reflective of P_C. If P_A and P_V did not exactly equilibrate to the same pressure upon double occlusion, then the mean of both pressures was determined and was defined as P_C (20) (Fig. 36; Appendix B). Results of other studies have shown that P_C measured by double vascular occlusion is equivalent to P_C measured by other classical means, such as the micropuncture technique (77,107).

Protocol (Expt D): Animals were allowed to stabilize for 30 min before any hemodynamic variables were measured. ADM (9 ng/ml) was then infused into the splenic artery at a rate of 0.05 ml/min; this dose achieved approximately the same plasma concentration of ADM reaching the spleen as in Experiment C, given the difference in arterial blood flow. A double vascular occlusion was conducted 5 min after the infusion of ADM was started. The double occlusion was performed by simultaneously tightening a snare placed around the splenic vein (Fig. 1) while the perfusion pump was stopped and the tubing clamped, so that arterial inflow was blocked for a period of ~5 seconds (107) (Fig. 36; Appendix B). Control animals, implanted with the same cannulae and treated in the same manner as the experimental animals, were infused with saline and subjected to the same protocol.

The circulation of blood through the spleen may be represented by a simple linear model where P_A is separated from P_C by a pre-capillary resistance (R_A), and P_C is separated

from P_V by a post-capillary resistance (R_V) (20). The pre- and post-capillary resistances may be calculated using the following equations:

$R_A = (P_A - P_C)/Q$ and $R_V = (P_C - P_V)/Q$, where Q is equal to flow (ml/min).

Statistical analysis (Expt D): The significance of the saline versus ADM-induced alterations in pre-and post-capillary resistances, and intrasplenic P_C was assessed using the Student's t-test for unpaired data. The level of statistical significance was defined at $p< 0.05$.

EXPERIMENT E: EFFECT OF ADRENOMEDULLIN (ADM), ATRIAL NATRIURETIC FACTOR (ANF), ENDOTHELIN-1 (ET-1), SODIUM NITROPRUSSIDE (SNP) AND S-NITROSO-N-ACETYL-D,L-PENICILLAMINE (SNAP) ON VASOREACTIVITY OF ISOLATED NORMAL SPLENIC RESISTANCE ARTERIES AND VEINS.

Vessel preparation (Expt E): Rats were decapitated. The spleen and its associated vascular arcade were rapidly removed through a midline laparotomy, and placed in ice-cold HEPES-buffered phosphate saline solution (HEPES-PSS). Hilar arteries (125-200 μm) and veins (350-450 μm), that led directly into or out from the splenic tissue itself, were dissected free from surrounding adipose tissue, cut into ~2 mm lengths, and mounted on an isometric tension myograph system (Kent Scientific Corp., Litchfield, CA, USA). The blocks were positioned in an organ bath with 5 ml of HEPES-PSS solution kept at 37°C. Two separate organ baths were used to study matched arterial and

venous segments, and changes in isometric force were recorded on a data acquisition system (Windaq, DATAQ Instruments Inc, Akron, Ohio).

Resting length-tension curve (Expt E): After mounting, vessels were allowed to stabilize for 30 min in HEPES-PSS buffer under no tension, during which time the buffer solution was changed at 10 min intervals. This was followed by a preconditioned stretch of approximately 0.6 mN, after which vessels were rested at 0.1-0.2 mN/mm and allowed to stabilize in HEPES-PSS buffer for a further 10 min. This was followed by generation of a resting length-tension curve. From Laplace's law, the L_{100} or L_5 was calculated from the exponential curve fit of tension generated versus internal vessel circumference. The L_{100} is the circumference that an artery would have at a transmural pressure of 100 mmHg, and L_5 is the circumference that a vein would have at a transmural pressure of 5 mmHg. Preliminary studies on hilar arteries and veins indicated that the point on the passive-active tension characteristics curve obtained at $0.65L_{100}$ and $0.8L_5$ provided the maximum active tension with least passive tension for arteries and veins, respectively (Appendix C). Vessels were set to their determined optimal tension and allowed to stabilize for 30 minutes in HEPES-PSS buffer, with buffer changed at 10 min intervals, prior to generation of a dose response curve.

Solutions and drugs (Expt E): The HEPES-PSS solution which was maintained at a pH of 7.4, contained (mM) sodium chloride 142, potassium chloride 4.7, magnesium sulphate 1.17, calcium chloride 1.56, potassium phosphate 1.18, HEPES 10, and glucose 5.5. Stock solutions of L-phenylephrine hydrochloride and SNP (Sigma Chemical Co,

Ontario, Canada) and SNAP (World Precision Instruments, Sarasota, FL, USA) were prepared in water at concentrations of 10M, 1.68×10^{-2} M and 4.5×10^{-3} M, respectively. Appropriate dilutions of all stocks were obtained using HEPES-PSS.

Protocol (Expt E): The optimal tension was set for each vessel prior to assessing the vasoconstrictor or vasodilator responses. A single vessel was used per dose-response measurement. After a 30 min stabilization period, during which time the organ bath was changed with fresh buffer every 10 min, vessels were used for subsequent determination of vasoconstrictor responses to ANF (1×10^{-12}-1×10^{-6} M) or ET-1 (1×10^{-11}-1×10^{-7} M). Vessels were treated for 4 min at each cumulative dose of ANF, and 10 min for each cumulative dose of ET-1. Preliminary experiments confirmed that the maximal vasorelaxant response at each dose was achieved by this time for each vasoactive agent. For vessels used to assess the vasorelaxant response to ADM and the NO donor agents, a cumulative dose response curve to phenylephrine (1×10^{-8}-1×10^{-3} M) was first generated. The dose of phenylephrine needed to achieve an 80% maximal constriction (EC_{80}) over this concentration range was chosen (Fig. 44; Appendix I), as the preconstrictor value for both hilar arteries and veins, in order to assess the vasorelaxant properties of vessels that had the same *degree* of preconstrictor tone relative to maximal constriction for that vessel. However, it is acknowledged that the absolute tension would be greater in hilar arteries than veins. After a 30 min stabilization period, during which time the organ bath was changed with fresh buffer every 10 min, the determined EC_{80} to phenylephrine was used to preconstrict the vessel for subsequent vasorelaxant responses to SNP (1×10^{-12}-1×10^{-4} M) or SNAP (1×10^{-10}-3×10^{-4} M). Vessels were treated either with ADM, SNP or

SNAP for 4 min periods at each cumulative dose. Preliminary experiments confirmed that the maximal vasorelaxant response at each dose was achieved by this time.

Statistical analysis (Expt E): The significance of changes in tension at each of the cumulative doses for each vasoactive agent was analyzed by the one-way repeated measures ANOVA, followed by the Student-Newman-Keuls method to identify the individual points of significance. Where data were not normally distributed, a repeated measures ANOVA on ranks was used. The significance of differences between arteries and veins at particular doses, was analyzed by a two-way repeated measures ANOVA. The significance of differences between hilar arteries and veins, in the EC_{50} concentration of the dose-responses to ANF and ET-1, was determined by a Mann-Whitney rank sum test as the data was not normally distributed. The significance of differences in maximal vasorelaxation to ADM, SNP and SNAP between arteries and veins was determined by a Student's t-test for unpaired data. The significance of differences in maximal vasorelaxation between arteries and veins treated with SNP and SNAP was determined by a one-way ANOVA plus Dunn's test for multiple comparisons. Significance was accepted at $p<0.05$.

EXPERIMENT F: EFFECT OF N^G-MONOMETHYL-L-ARGININE (L-NMMA) ON ADRENOMEDULLIN (ADM)-INDUCED RELAXATION OF ISOLATED NORMAL SPLENIC RESISTANCE ARTERIES.

Vessel preparation (Expt F): Splenic hilar arteries and hilar veins were obtained in an identical manner as described in experiment E. After characterization of the active-passive tension curves for each vessel, a cumulative dose response curve to phenylephrine ($1x10^{-8}$-$1x10^{-3}$ M) was generated. From this curve the dose required to achieve 80% maximal constriction (EC_{80}) to phenylephrine was determined. The effect of NO synthase inhibition was investigated by incubating the vessels with N^G-monomethyl-L-arginine (L-NMMA) (10^{-4} M) or the inactive enatiomer N^G-monomethyl-D-arginine (D-NMMA) (10^{-4} M) (Calbiochem, LaJolla, CA., USA) for 15 min before pre-constriction and construction of the ADM dose-response curve. After a 30 min stabilization period, during which time the organ bath was changed with fresh buffer every 10 min, phenylephrine (the EC_{80} concentration) was used to pre-constrict the vessel for determination of the vasodilatory response to ADM ($1x10^{-11}$-$1x10^{-6}$ M).

Statistical analysis (Expt F): The significance of changes in tension at each of the cumulative doses for hilar arteries treated with ADM plus D-NMMA and those hilar arteries treated with ADM plus L-NMMA was analyzed by a one-way repeated measures ANOVA, followed by the Student-Newman-Keuls method to identify the individual points of significance. Where data were not normally distributed, a repeated measures ANOVA on ranks was used. The significance of differences between each of these treated groups at a particular dose of ADM was analyzed by the two-way repeated measures ANOVA. The significance of differences in maximal vasorelaxation between ADM plus D-NMMA versus ADM plus L-NMMA treated hilar arteries was determined by a Student's t-test for unpaired data. Significance was accepted at $p<0.05$.

EXPERIMENT G: EFFECT OF PHENYLEPHRINE, ENDOTHELIN-1 (ET-1), S-NITROSO-N-ACETYL-D,L-PENICILLAMINE (SNAP) AND ADRENOMEDULLIN (ADM) ON VASOREACTIVITY OF ISOLATED LIPOPOLYSACCHARIDE (LPS)-EXPOSED (150μg/kg/hr for 3 hr *in vivo*) SPLENIC RESISTANCE ARTERIES AND VEINS.

Vessel preparation (Expt G): Rats were prepared as in the *in vivo* experiments, but in this instance for infusion of LPS (150 μg/kg/hr) into the bloodstream for a period of 3 hr only. At the end of the 3 hr LPS infusion period the animal was decapitated. The spleen and its associated vascular arcade were rapidly removed and mounted on the wire myograph system as described in experiment E.

Resting length-tension curve (Expt G): Mounted vessels were treated as previously described in experiment E. Preliminary studies on 3 hr LPS-exposed hilar arteries and veins indicated that the point on the passive-active tension characteristics curve obtained at $0.65L_{100}$ and $0.8L_5$ provided the maximum active tension with least passive tension for arteries and veins, respectively.

Solutions and drugs (Expt G): These were prepared as described in experiment E.

Protocol (Expt G): The generation of cumulative dose-responses to phenylephrine, ET-1, ADM and SNAP were as previously described in experiment E.

Statistical analysis (Expt G): The significance of changes in tension at each of the cumulative doses for phenylephrine, ET-1, ADM and SNAP was analyzed by a one-way repeated measures ANOVA, followed by the Student-Newman-Keuls method to identify the individual points of significance. Where data were not normally distributed, a repeated measures ANOVA on ranks was used. The significance of differences between arteries and veins at a particular concentration, was analyzed by a two-way repeated measures ANOVA. The significance of differences between 3 hr LPS-exposed hilar arteries and veins (and between the vessels used in experiment E) in the phenylephrine EC_{80} concentration was determined by a one-way ANOVA plus Dunn's test for multiple comparisons. The significance of differences between 3 hr LPS-exposed hilar arteries and veins (and between the vessels used in experiment E) in the EC_{50} concentration of ET-1 was determined by a Mann-Whitney rank sum test, as the data was not normally distributed. The significance of differences in maximal vasorelaxation to ADM and SNAP between 3 hr LPS-exposed hilar arteries and veins versus the vessels used in experiment E was determined by a one-way ANOVA plus Dunn's test for multiple comparisons. Significance was accepted at $p<0.05$.

CHAPTER 3.

RESULTS

EXPERIMENT A: EFFECT OF SPLENECTOMY ON LIPOPOLYSACCHARIDE (LPS)-INDUCED HYPOTENSION, HEMOCONCENTRATION AND HYPOVOLEMIA IN CONSCIOUS MALE RATS.

There were no significant differences between the basal measurements of MAP in any of the four groups of animals, nor were there any changes in MAP in either the intact or splenectomized, saline-infused groups (Fig. 13A). However, LPS infusion caused a significant decrease in MAP throughout the duration of the experimental period in the intact animals, relative both to the basal values and compared with the saline-infused intact group (Fig. 13B). Splenectomy abolished this response i.e. there was no significant change in MAP in the splenectomized rats infused with LPS compared with the saline-infused splenectomized group. Blood pressure was significantly lower in the intact rats than in the splenectomized rats at all points following initiation of the LPS infusion.

There were no significant differences between the basal values of hematocrit in any of the four groups of animals, nor were there any significant changes in hematocrit in either the intact or splenectomized, saline-infused groups (Fig. 14A). In the intact animals, LPS infusion resulted in a significant increase in hematocrit at 20 min, 40 min, 60 min, 90 min post-LPS infusion relative both to the basal/0 values and compared to the saline-infused, intact control group (Fig. 14B). By contrast, LPS infusion failed to induce any change in hematocrit in the splenectomized group. Hematocrit was significantly higher in the intact rats than in the splenectomized rats at 20, 40, 60 and 90 min following initiation of the LPS infusion.

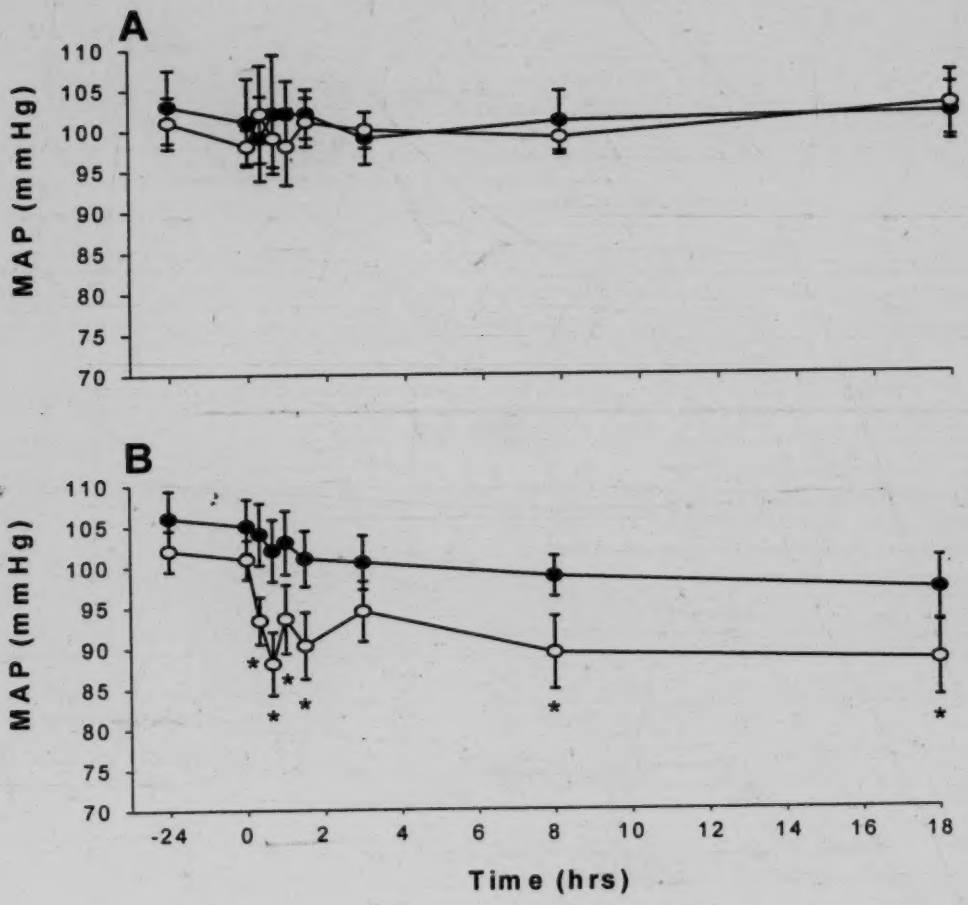

Figure 13. Mean arterial pressure (MAP) in conscious intact (open circles, n= 7) and splenectomized rats (closed circles, n=9) infused with A) saline and B) lipopolysaccharide (LPS) (150 μg/kg/hr). Vertical bars delineate standard error of the mean. *, Significant difference between intact and splenectomized LPS-infused rats and between intact LPS-infused group and its respective saline-infused control group, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

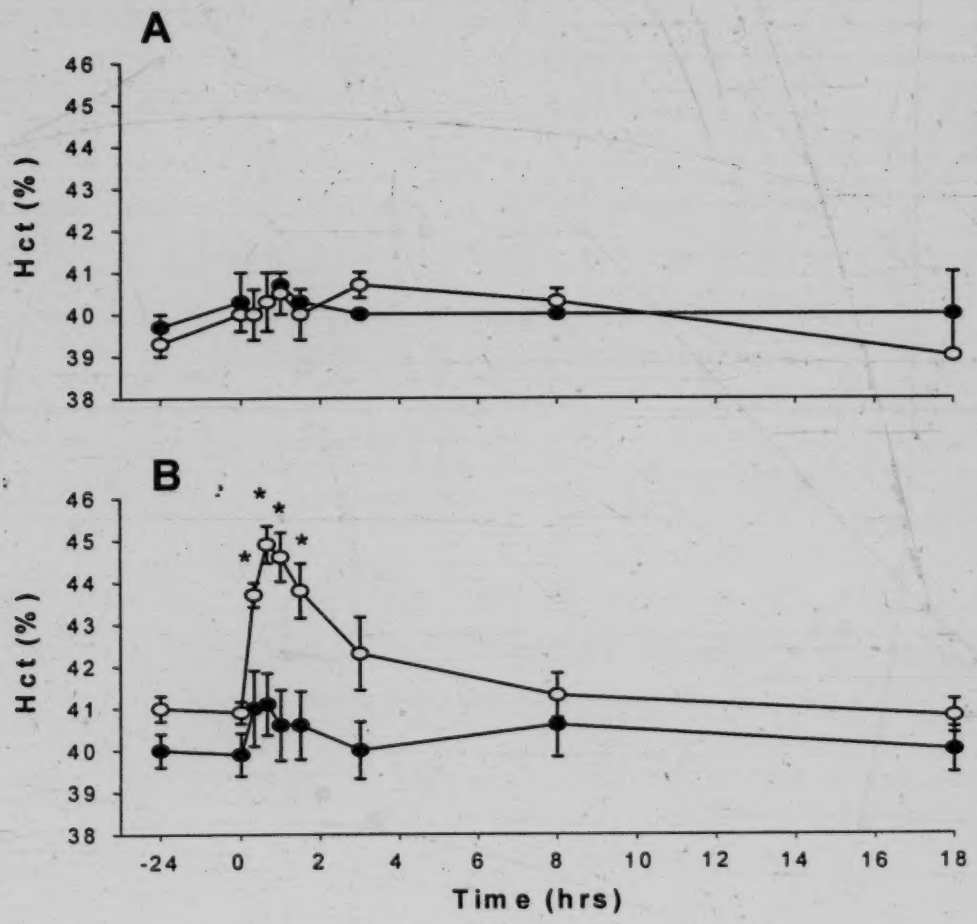

Figure 14. Hematocrit (Hct) in conscious intact (open circles, n= 7) and splenectomized rats (closed circles, n=9) infused with A) saline and B) lipopolysaccharide (LPS) (150 μg/kg/hr). Vertical bars delineate standard error of the mean. *, Significant difference between intact and splenectomized LPS-infused rats and between intact LPS-infused group and its respective saline-infused control group, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

There were no significant differences between the basal values of heart rate in any of the four experimental groups, nor were there any significant changes in heart rate in either the intact or splenectomized, saline-infused groups (Fig. 15). There was a significantly greater tachycardia in the intact LPS-infused group compared to the splenectomized LPS-infused group and its respective saline-infused control at 60 min, 90 min, 8 hr and 18 hr post-LPS infusion (*, $p<0.05$) (Fig. 15).

After 90 min LPS infusion, plasma volume was significantly reduced in the intact animals, but remained unchanged in the splenectomized group (Fig. 16). During the period of determination of plasma volume (between 90-140 min post-LPS infusion), there was no significant change in hematocrit in saline-infused controls ($p>0.05$). It may thus be assumed that plasma volume was in a steady state at this time.

There were no significant differences between the basal values of plasma protein concentration in any of the four experimental groups, nor were there any significant changes in plasma protein concentration in either the intact or splenectomized, saline-infused groups (Fig. 17A). In both the intact and splenectomized rats, plasma protein concentration significantly decreased at 3 hr, 8 hr and 18 hr post-LPS infusion in comparison to their relative basal/saline-infused control values (Fig. 17B) ($p<0.05$). There was no significant change in plasma protein concentration in the early hours of LPS infusion in either group (between 0-3 hr post-LPS infusion).

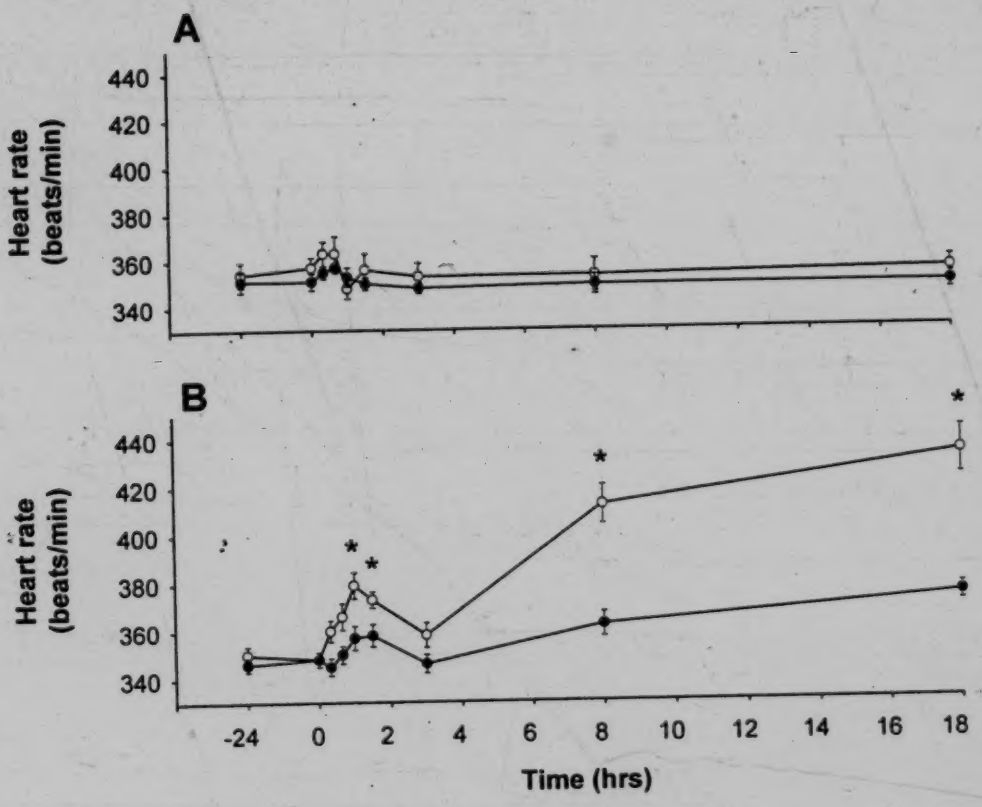

Figure 15. Heart rate in conscious intact (open circles, n= 7) and splenectomized rats (closed circles, n=7) infused with A) saline and B) lipopolysaccharide (LPS) (150 μg/kg/hr). Vertical bars delineate standard error of the mean. *, Significant difference between intact and splenectomized LPS-infused rats and between intact LPS-infused group and its respective saline-infused control group, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

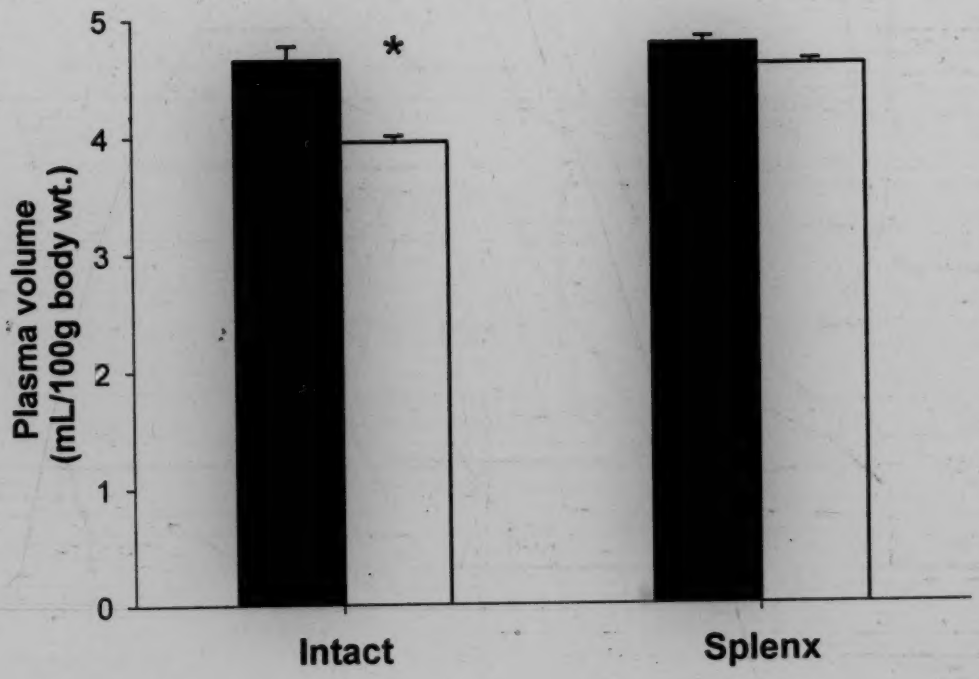

Figure 16. Plasma volume of intact (n=4) and splenectomized (splenx, n=4) rats before (solid bars) and after (open bars) 90 min infusion of lipopolysaccharide (LPS) (150 μg/kg/hr) into conscious rats. Vertical bars delineate standard error of the mean. *, Significant difference between intact and splenectomized LPS-infused rats and between intact LPS-infused group and its respective saline-infused control group, $p<0.05$. One-way ANOVA on ranks plus Dunn's test for multiple comparisons.

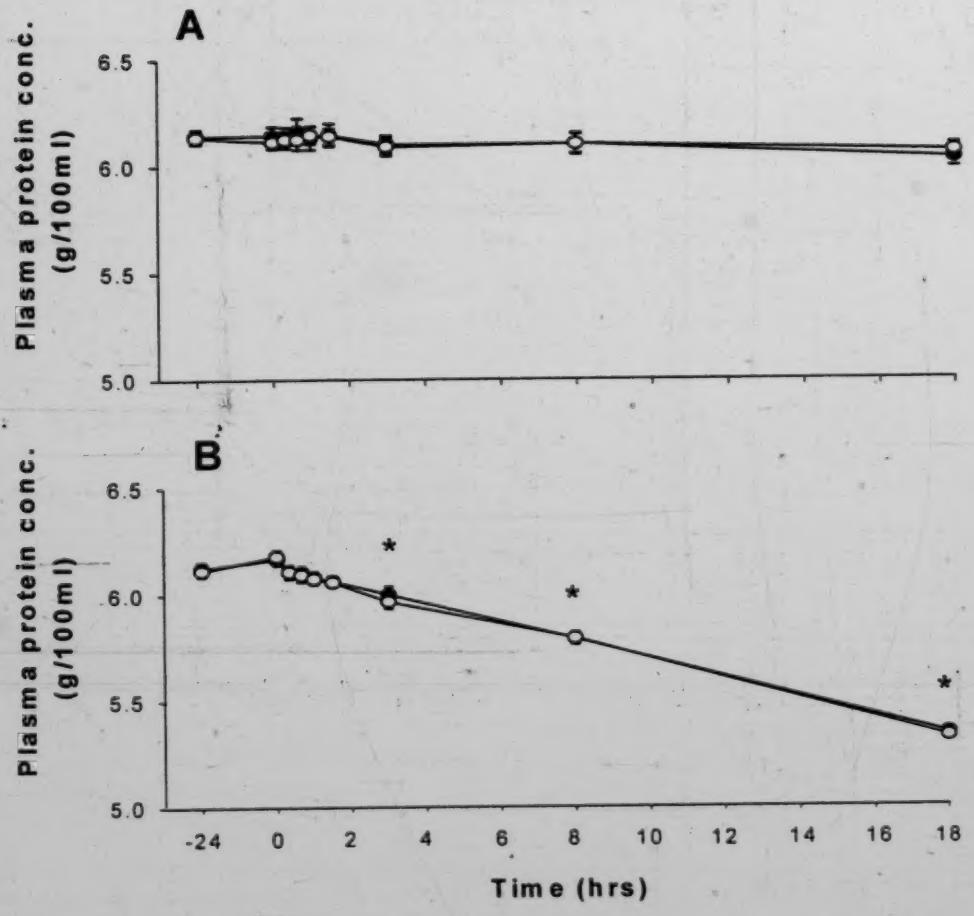

Figure 17. Plasma protein concentration in conscious intact (open circles, n=7) and splenectomized rats (closed circles, n=9) infused with A) saline and B) lipopolysaccharide (LPS) (150 μg/kg/hr). Vertical bars delineate standard error of the mean. *, Significant difference between saline-infused and LPS-infused rats from both intact and splenectomized groups, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

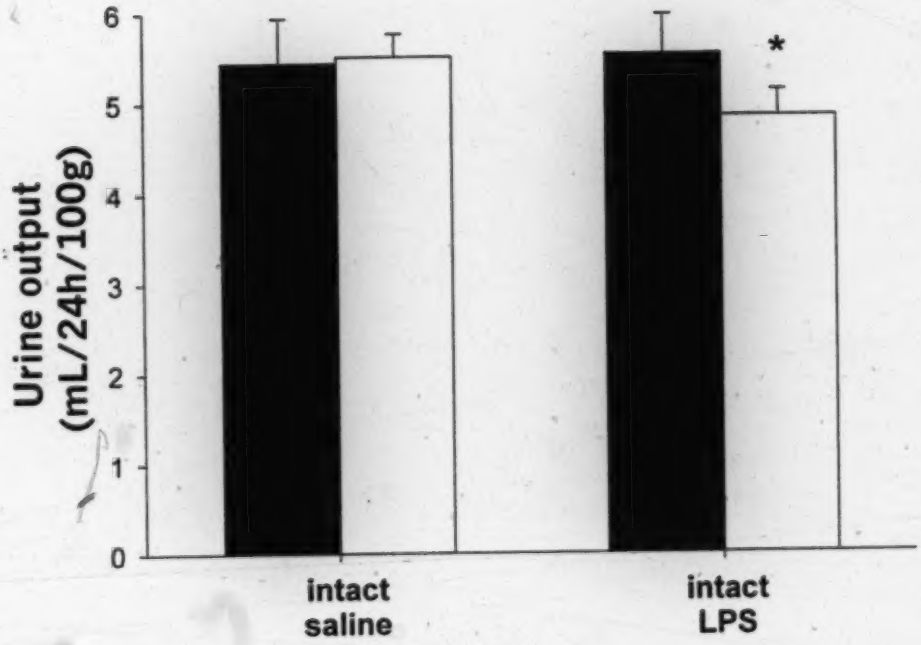

Figure 18. Average volume of urine output in intact conscious rats, 24 hours before infusion period (basal values) (solid bars) and over 24 hours of either lipopolysaccharide (LPS) (150 μg/kg/hr) (n=7) or saline infusion (open bars) (n=7). Vertical bars delineate standard error of the mean. *, Significant difference between intact saline-infused and LPS-infused rats and between intact LPS-infused group and respective basal value, $p<0.05$. One-way ANOVA on ranks plus Dunn's test for multiple comparisons.

The average volume of urine output in intact conscious rats over the 24 hr period prior to infusion of either saline or LPS were not significantly different ($p>0.05$). There was a significant decrease in the average volume of urine output between the intact LPS-infused group and its respective basal value/saline-infused control over the 24 hr infusion period ($p<0.05$) (Fig. 18).

EXPERIMENT B: EFFECT OF SPLENIC DENERVATION ON LIPOPOLYSACCHARIDE (LPS)-INDUCED HYPOTENSION, HEMOCONCENTRATION AND HYPOVOLEMIA IN CONSCIOUS MALE RATS.

There were no significant differences between the intact and denervated groups in the resting (time=0) splenic arterial or venous blood flows, or in the resting (time = 0) A-V flow differential (fluid efflux) (Fig. 19) ($p>0.05$). Nor were there any significant changes in splenic blood flows or A-V flow differential over the course of 18 hours in the respective saline-infused control groups (Fig. 40; Appendix E) ($p>0.05$). Following infusion of LPS, splenic arterial blood flow increased significantly in both intact and denervated groups with regard to both basal values and their respective saline-infused control group (Fig. 19A) (§, $p<0.05$). The LPS-induced increase in splenic arterial blood flow was significantly greater in the denervated group than the intact group at 60 min, 90 min and 3 hr post-infusion (Fig. 19A) (*, $p<0.05$). There was no significant difference in venous blood flow between denervated and intact groups infused with LPS (Fig. 19B) ($p>0.05$). The A-V flow differential significantly increased in response to LPS in both the denervated and intact groups, both with regard to basal values and their respective saline-infused control group (Fig. 19C) (§, $p<0.05$). The LPS-induced rise in intrasplenic fluid

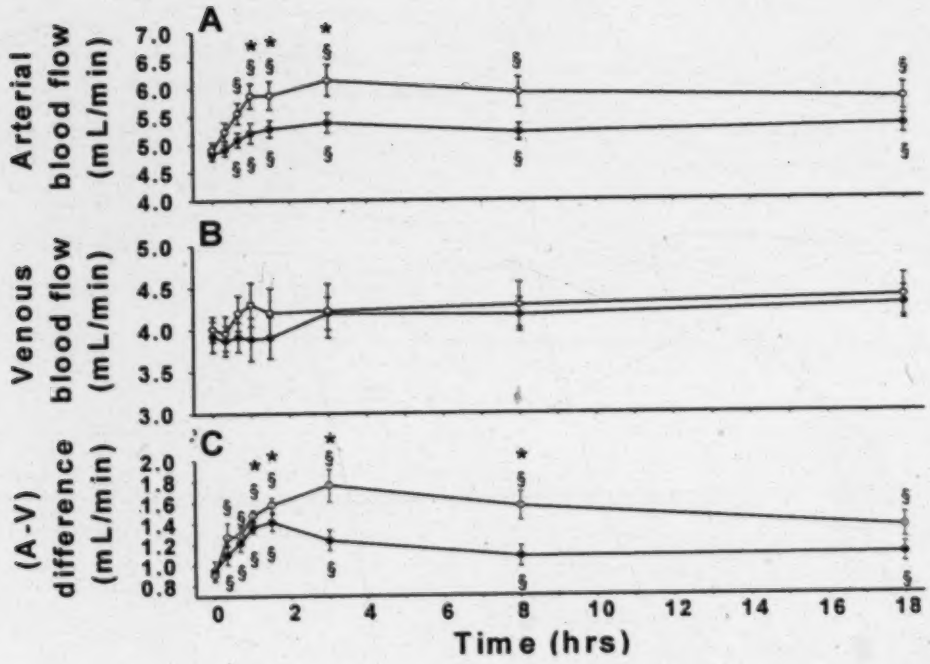

Fig. 19. Splenic A) arterial and B) venous blood flows in denervated (open circles, n=10) and intact (solid circles, n=8) conscious rats during lipopolysaccharide (LPS) infusion (150μg/kg/hr), and C) fluid efflux (A-V difference) in denervated (open circles, n=8) and intact (solid circles, n=8) groups. Vertical bars delineate standard error of the mean. §, Significant difference between LPS-infused groups and their respective saline-infused control groups (Fig. 40; Appendix E) and between the LPS-infused groups and their respective pre-infusion (0 min) basal values, $p<0.05$. *, Significant difference between intact and denervated LPS-infused groups, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

efflux was significantly greater in the splenic denervated than the intact group at 60 min, 90 min, 3hr and 8 hr post-infusion (Fig. 19C) (*, $p<0.05$). The mean increase in intrasplenic fluid efflux over the 18 hr LPS infusion period was significantly greater in the denervated ($+0.41 \pm 0.08$ ml/min, n=7) than in the intact ($+0.21 \pm 0.06$ ml/min, n=8) group ($p<0.05$).

There was no significant difference in basal (-24 hr) MAP between the intact and denervated groups (Fig. 20A) ($p>0.05$), nor were there any significant changes in MAP over the course of 18 hours in the respective saline-infused controls (Fig. 41A; Appendix F) ($p>0.05$). A significant decrease in MAP occurred at all time points in both intact and denervated LPS-infused groups relative to their respective basal (-24 hr) and saline-infused control values (Fig. 20A) (§, $p<0.05$). MAP was significantly lower in the denervated LPS-infused group compared to the intact LPS-infused group at all time points except 90 min (Fig. 20A, 21B) (*, $p<0.05$).

There was no significant difference in basal (-24 hr) hematocrit between the intact and denervated groups (Fig. 20B) ($p>0.05$), nor were there any significant changes in hematocrit over the course of 18 hours in the respective saline-infused controls (Fig. 41B; Appendix F) ($p>0.05$). In response to LPS infusion, hematocrit increased in both the intact and denervated groups with regards to respective basal values and saline-infused controls (Fig. 20B) (§, $p<0.05$). However, the increase in hematocrit was significantly greater in the denervated group compared to the intact group at 90 min, 3 hr, and 8 hr post-LPS infusion (*, $p<0.05$).

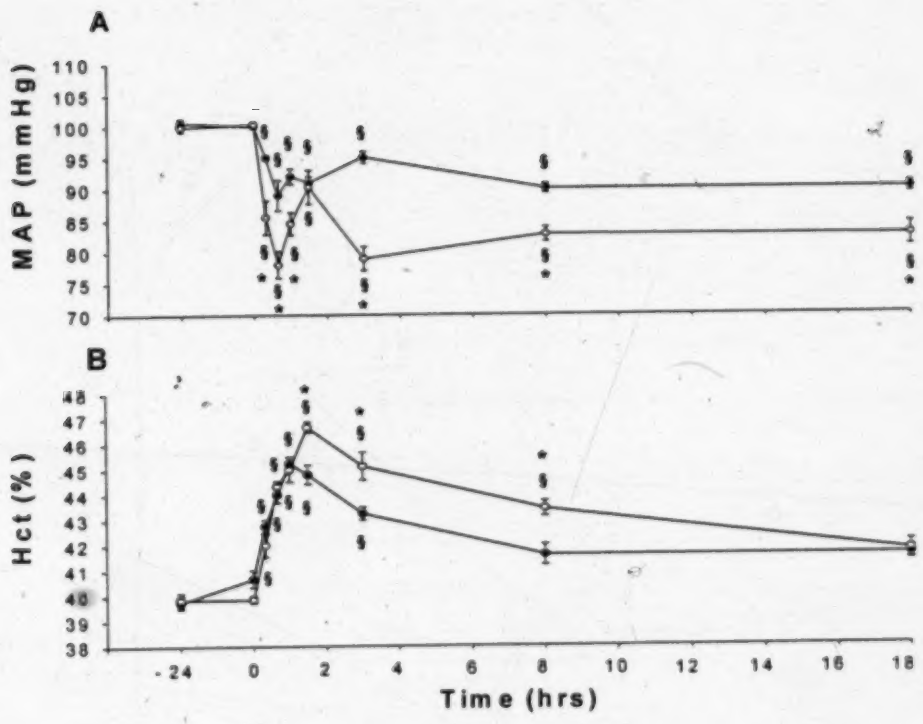

Figure 20. A) Mean arterial pressure (MAP) and B) hematocrit (Hct) during intravenous infusion of lipopolysaccharide (LPS) (150μg/kg/hr) in intact (solid circles, n=8) and splenic denervated (open circles, n=8) conscious rats. Vertical bars delineate standard error of the mean. §, Significant difference between LPS-infused groups (intact and denervated) and their respective basal values and saline-infused groups (Fig. 41; Appendix F). *, Significant difference between intact and denervated LPS-infused groups, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

There was no significant difference in basal (-24 hr) heart rate between the intact and denervated groups (Fig. 21D) ($p>0.05$), nor were there any significant changes in heart rate over the course of 18 hours in the respective saline-infused controls (data not shown) ($p>0.05$). In response to LPS infusion, heart rate increased in both the intact and denervated groups compared with their respective basal values and saline-infused controls (Fig. 21D; Fig. 42D; Appendix G) (§, $p<0.05$). However, the increase in heart rate was significantly greater in the denervated group compared to the intact group at 60 min, 90 min, and 3 hr post-LPS infusion (Fig. 21D) (*, $p<0.05$).

Basal (-24 hr) plasma volume was significantly greater in the splenic denervated group than the intact group (Fig. 22) (§, $p<0.05$). Following 90 min of LPS infusion there was a significant fall in plasma volume from respective basal control values in both intact (†, $p<0.05$) and denervated (@, $p<0.05$) groups (Fig. 22). There was a significant difference in plasma volume between the intact and denervated groups at 90 min post-LPS infusion (Fig. 22) (*, $p<0.05$). Moreover, the decrease in plasma volume from basal to 90 min post-LPS infusion was significantly greater in the denervated group (1.1 ± 0.2 ml/100g B. Wt, n=7) than the intact group (0.54 ± 0.1 ml/100g B. Wt, n=8) ($p<0.05$). The decrease in plasma volume is physiologically significant, given that the relative loss of intravascular fluid in both experimental groups is greater than 10% of the typical plasma volume of a rat (with plasma volume per animal body weight taken into account).

Basal (-24 hr) plasma renin activity (PRA) tended to be higher in the denervated saline-infused control group (7.5 ± 0.5 ng AI/ml/hr, n=7) than the intact saline-infused control

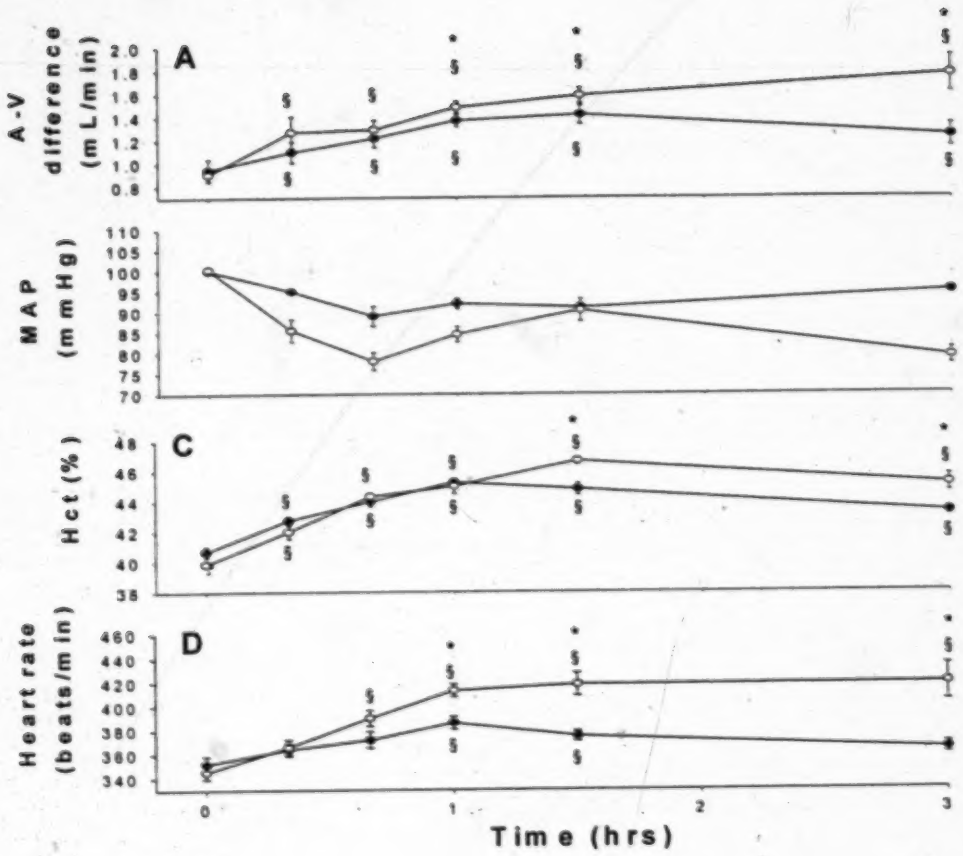

Figure 21. A) Splenic (A-V) difference B) mean arterial pressure (MAP) C) hematocrit (Hct) and D) heart rate during intravenous infusion of lipopolysaccharide (LPS) (150 μg/kg/hr) in intact (solid circles) and splenic denervated (open circles) conscious rats. Vertical bars delineate standard error of the mean. §, Significant difference between LPS-infused groups (intact and denervated) and their respective basal values and saline-infused groups (Fig. 42; Appendix G). *, Significant difference between intact and denervated LPS-infused groups, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

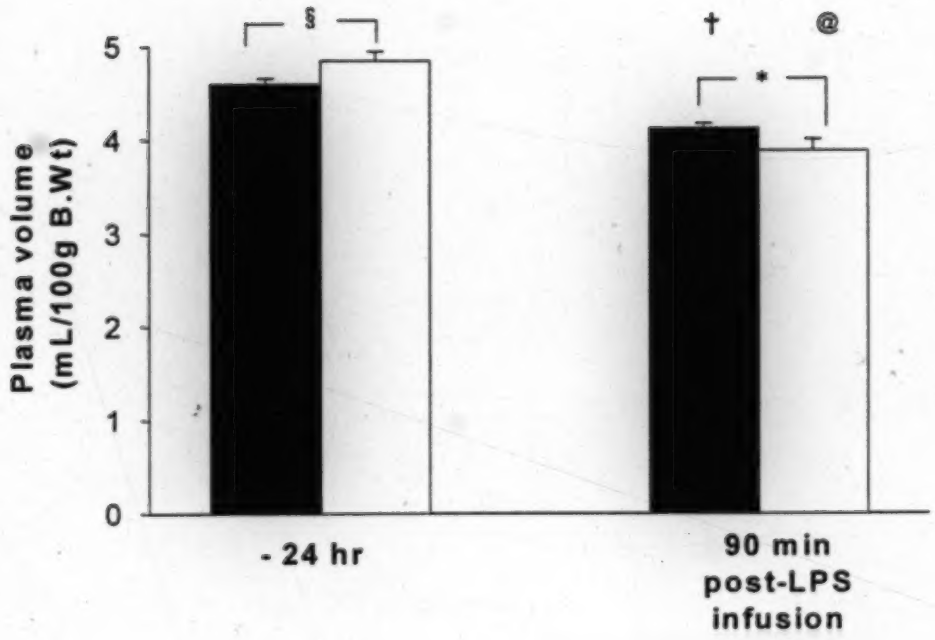

Figure 22. Plasma volume at basal (-24 hr) and 90 min post-infusion of lipopolysaccharide (LPS) (150μg/kg/hr) in intact (closed bars, n=8) and denervated (open bars, n=7) conscious rats. Vertical bars delineate standard error of the mean. †, Significant difference in plasma volume in intact rats at basal versus 90 min post-LPS infusion, $p<0.05$. @, Significant difference in plasma volume in denervated rats at basal versus 90 min post-LPS infusion, $p<0.05$. §, Significant difference in basal plasma volume between intact and denervated groups, $p<0.05$. *, Significant difference in plasma volume at 90 min post-LPS infusion between intact and denervated LPS-infused groups, $p<0.05$. One-way ANOVA on ranks plus Dunn's test for multiple comparisons.

group (4.7±0.3 ng AI/ml/hr, n=7) ($p=0.323$). There was no significant change in PRA over the 18 hr period of saline infusion in intact or denervated saline-infused controls (Fig. 43; Appendix H) ($p>0.05$). LPS caused a significant time-dependent increase in PRA in both intact (@, $p<0.05$) and denervated (†, $p<0.05$) groups (Fig. 23). Moreover, the increase in PRA was significantly greater in the denervated group than in the intact group at 90 min, 8 hr, and 18 hr post-LPS infusion (Fig. 23) (*, $p<0.05$). The intra-assay and inter-assay variability of the PRA measurements were 6 % and 11 %, respectively.

Total catecholamine content of splenic tissue at 18 hr post-saline/LPS infusion was significantly less in denervated (32 ± 8 ng/ml, n=10) than intact (222 ± 34 ng/ml, n=10) groups ($p<0.05$). The intra-assay variability of the catecholamine measurements was 2 %.

Basal (-24 hr) plasma TNF-α concentration in the denervated and intact groups was below the detection limit of the assay. There was no detectable change in plasma TNF-α concentration in either intact or denervated control groups at 90 min and 3 hr post-saline infusion. LPS caused a significant increase in plasma TNF-α concentration in both intact (90 min: 2244 ± 654 pg/ml; 3 hr: 1606 ± 113 pg/ml, n=4) and denervated (90 min: 2231 ± 532 pg/ml; 3 hr: 2006 ± 577 pg/ml, n=4) groups compared to their respective basal value and saline-infused controls ($p<0.05$). Plasma TNF-α concentrations were not significantly different between the denervated and intact groups at 90 min or 3 hr post-LPS infusion ($p>0.05$). The intra-assay variability of the plasma TNF-α measurements was 5 %.

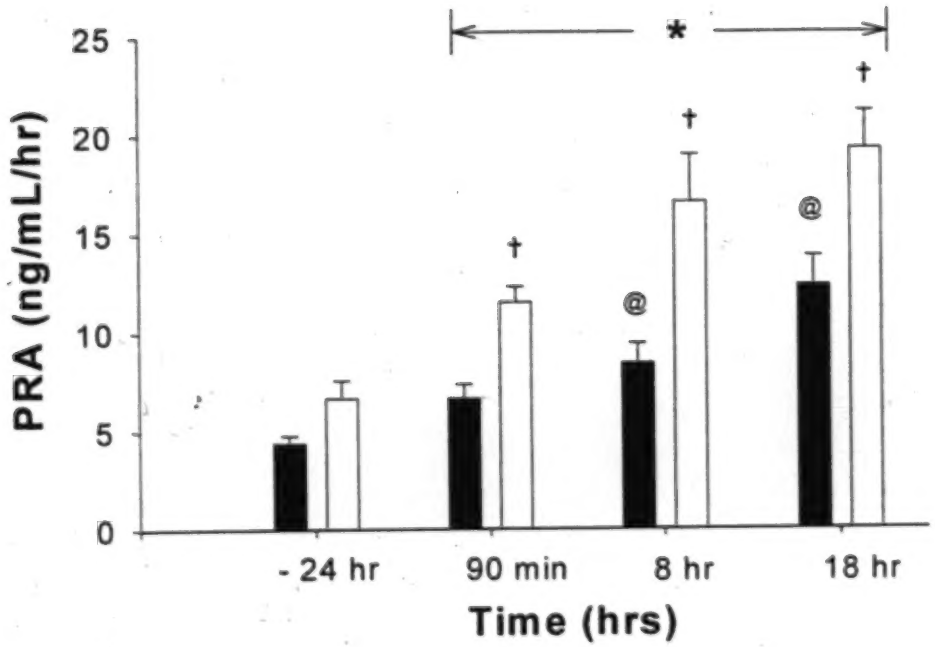

Figure 23. Plasma renin activity (PRA) before (-24 hr) and during intravenous infusion of lipopolysaccharide (LPS) (150μg/kg/hr) in intact (closed bars, n=8) and splenic denervated (open bars, n=9) conscious rats. Vertical bars delineate standard error of the mean. @, Significant difference between intact LPS-infused group and its saline-infused control group (Fig. 43; Appendix H), $p<0.05$. †, Significant difference between denervated LPS-infused group and its saline-infused control group (Fig. 43; Appendix H), $p<0.05$. *, Significant difference between intact and denervated LPS-infused groups, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

There were no significant differences in the wet weight of splenic tissue at 18 hr post-infusion between intact saline-infused (1.27 ± 0.1 g, n=13), intact LPS-infused (1.28 ± 0.1 g, n=10), denervated saline-infused (1.27 ± 0.1 g, n=11), and denervated LPS-infused (1.26 ± 0.1 g, n=13) groups ($p>0.05$).

EXPERIMENT C: EFFECT OF INTRASPLENIC INFUSION OF ADRENOMEDULLIN (ADM) AND S-NITROSO-N-ACETYL-D,L-PENICILLAMINE (SNAP) ON SPLENIC ARTERIAL VERSUS VENOUS HEMATOCRIT AND WET SPLENIC TISSUE WEIGHT IN ANESTHETIZED MALE RATS.

There was an ADM-induced increase in splenic venous hematocrit (saline: 42.0 ± 0.3%, n=6 versus ADM-infused: 43.9 ± 0.3%, n=7) compared to arterial hematocrit (saline: 41.2 ± 0.2%, n=6 versus ADM-infused: 40.7 ± 0.2%, n=7) ($p<0.05$), but no change in wet splenic tissue weight (ADM-infused: 1.0 ± 0.01g, n=7 versus saline-infused: 0.99 ± 0.03g, n=6) ($p>0.05$) i.e. there was an ADM-induced increase in loss of cell-free fluid within the splenic circulation. There was a SNAP-induced increase in splenic venous hematocrit (saline: 41.0 ± 0.4%, n=3 versus SNAP-infused: 43.5 ± 0.5%, n=3) compared to arterial hematocrit (saline: 40.0 ± 0.3%, n=3 versus SNAP-infused: 41.0 ± 0.4%, n=3) ($p<0.05$), but no change in wet splenic tissue weight (SNAP-infused: 1.1 ± 0.05g, n=3 versus saline-infused: 1.0 ± 0.05g, n=3) ($p>0.05$) i.e. there was a SNAP-induced increase in loss of cell-free fluid within the splenic circulation.

EXPERIMENT D: EFFECT OF ADRENOMEDULLIN (ADM) ON INTRASPLENIC MICROVASCULAR PRESSURE.

In the isolated, blood-perfused spleen, intrasplenic infusion of ADM resulted in a significant decrease in pre-capillary resistance (saline-infused: 95 $\pm$ 1.3 mmHg/ml, n=5 versus ADM-infused: 89 $\pm$ 1.4 mmHg/ml, n=6) ($p<0.05$), no change in post-capillary resistance (saline-infused: 9 $\pm$ 0.3 mmHg/ml, n=5 versus ADM-infused: 10 $\pm$ 0.4 mmHg/ml, n=6) ($p>0.05$), and a significant increase in intrasplenic P_C (saline-infused: 11.0 $\pm$ 0.3 mmHg, n=5 versus ADM-infused: 13.4 $\pm$ 0.2 mmHg, n=6) ($p<0.05$).

EXPERIMENT E: EFFECT OF ADRENOMEDULLIN (ADM), ATRIAL NATRIURETIC FACTOR (ANF), ENDOTHELIN-1 (ET-1), SODIUM NITROPRUSSIDE (SNP), AND S-NITROSO-N-ACETYL-D,L-PENICILLAMINE (SNAP) ON VASOREACTIVITY OF ISOLATED NORMAL SPLENIC RESISTANCE ARTERIES AND VEINS.

There was a significant concentration-dependent ($1x10^{-8}$ to $1x10^{-3}$ M) increase in tension to phenylephrine in both hilar arteries (n=9) and hilar veins (n=11) ($p<0.05$). The maximal tension generated at the highest dose of phenylephrine ($1x10^{-3}$ M) was 2.1 $\pm$ 0.1 mN/mm and 0.5 $\pm$ 0.03 mN/mm for hilar arteries and veins, respectively (Fig. 44; Appendix I). There was no significant difference in the phenylephrine EC_{80} values between hilar arteries (4.86×10^{-6} M $\pm$ 0.9×10^{-6} M) and veins (6.44×10^{-6} M $\pm$ 1.2×10^{-6} M) ($p<0.05$) (Fig. 45; Appendix J) i.e. although the individual pre-constrictive EC_{80}

doses used for vessels varied slightly, the mean EC_{80} dose of phenylephrine applied to arteries was not significantly different from that used for veins. This 80% preconstriction value (EC_{80}) was chosen in order to assess the vasorelaxant properties of vasoactive agents on splenic resistance vessels that had the same *degree* of preconstrictor tone relative to maximal vasoconstriction for that vessel.

ADM caused a dose-dependent relaxation of phenylephrine-preconstricted splenic resistance arteries and veins (Fig. 24). However, the maximal relaxation was significantly greater in the hilar arteries ($60 \pm 1\%$, n=9) than in hilar veins ($43 \pm 2\%$, n=8) ($p<0.05$) i.e. the vasorelaxant efficacy of ADM was greater in the arteries than in the veins.

ANF caused a dose-dependent vasoconstriction of both splenic resistance arteries and veins ($p<0.05$) (Fig. 25). The potency of ANF was significantly greater on the veins than the arteries (EC_{50}= artery 8.1×10^{-9} M $\pm$ 1.6×10^{-9} M versus vein 3.2×10^{-9} M $\pm$ 1.0×10^{-9} M) ($p<0.05$).

ET-1 caused a dose-dependent vasoconstriction of both splenic resistance arteries and veins ($p<0.05$) (Fig. 26). The potency of ET-1 was significantly greater on the veins than the arteries (EC_{50}= artery 3.7×10^{-9} M $\pm$ 4.5×10^{-9} M versus vein 6.8×10^{-10} M $\pm$ 2.4×10^{-10} M) ($p<0.05$).

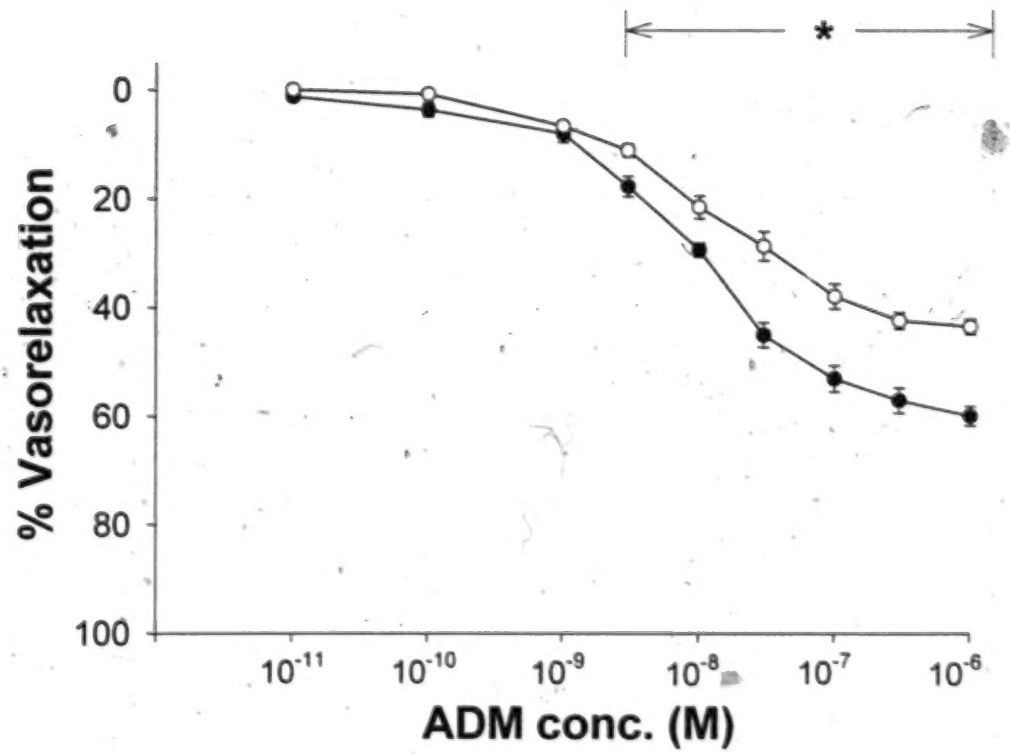

Figure 24. Effect of adrenomedullin (ADM) on vasorelaxation of splenic resistance arteries (closed circles, n=9) and veins (open circles, n=8). The vessels were preconstricted with phenylephrine (EC_{80}). *, Significant difference between % vasorelaxation of artery and vein, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

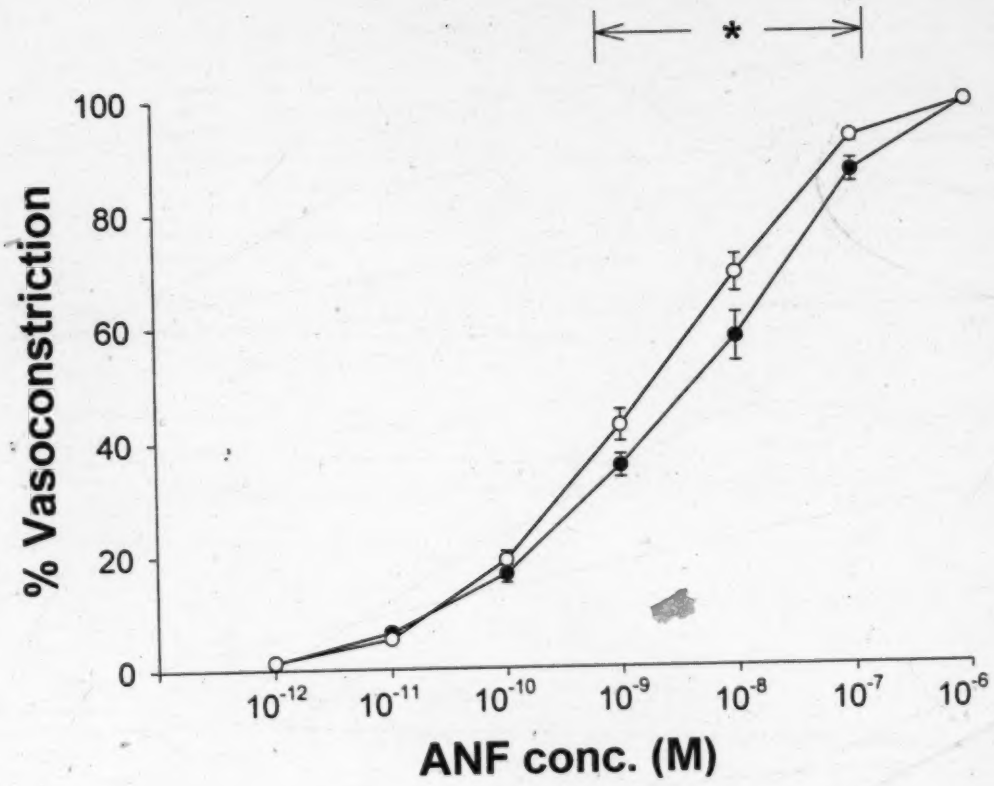

Figure 25. Vasoconstrictor effect of atrial natriuretic factor (ANF) on splenic resistance arteries (closed circles, n=9) and veins (open circles, n=9). *, Significant difference between % vasoconstriction of artery and vein, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

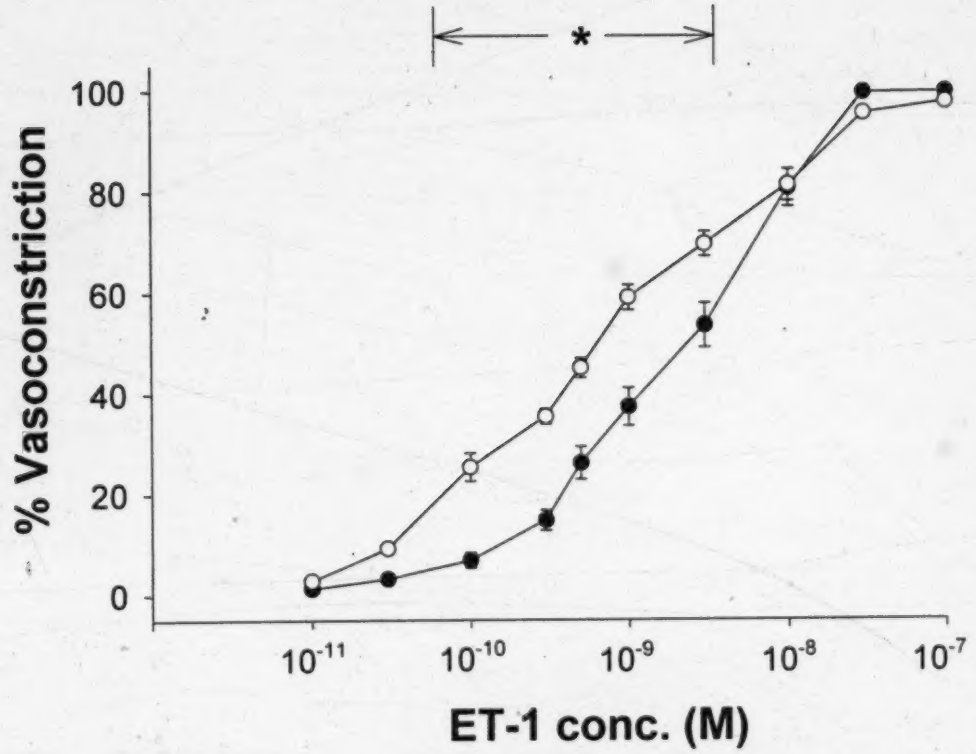

Figure 26. Vasoconstrictor effect of endothelin-1 (ET-1) on splenic resistance arteries (closed circles, n=9) and veins (open circles, n=8). *, Significant difference between % vasoconstriction of artery and vein, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

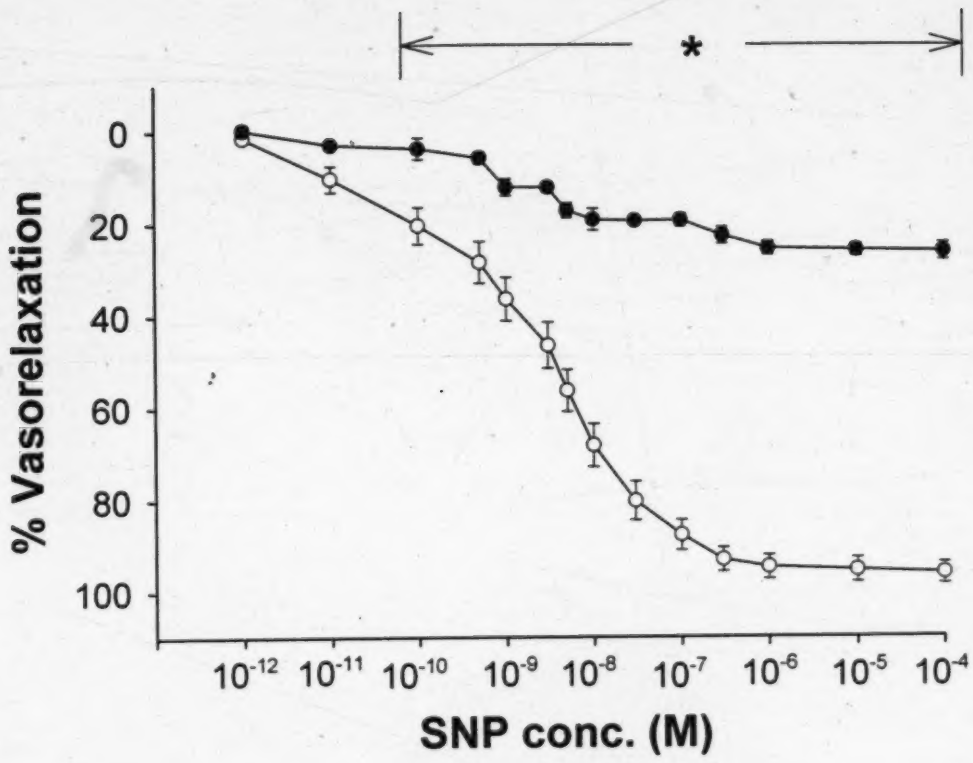

Figure 27. Vasorelaxant effects of sodium nitroprusside (SNP) on hilar arteries (closed circles, n=9) and veins (open circles, n=10). Cumulative dose-response curves to SNP, following preconstriction with phenylephrine (EC_{80}). Vertical bars delineate standard error of the mean. *, Significant difference between splenic resistance artery and vein ($p<0.05$). Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

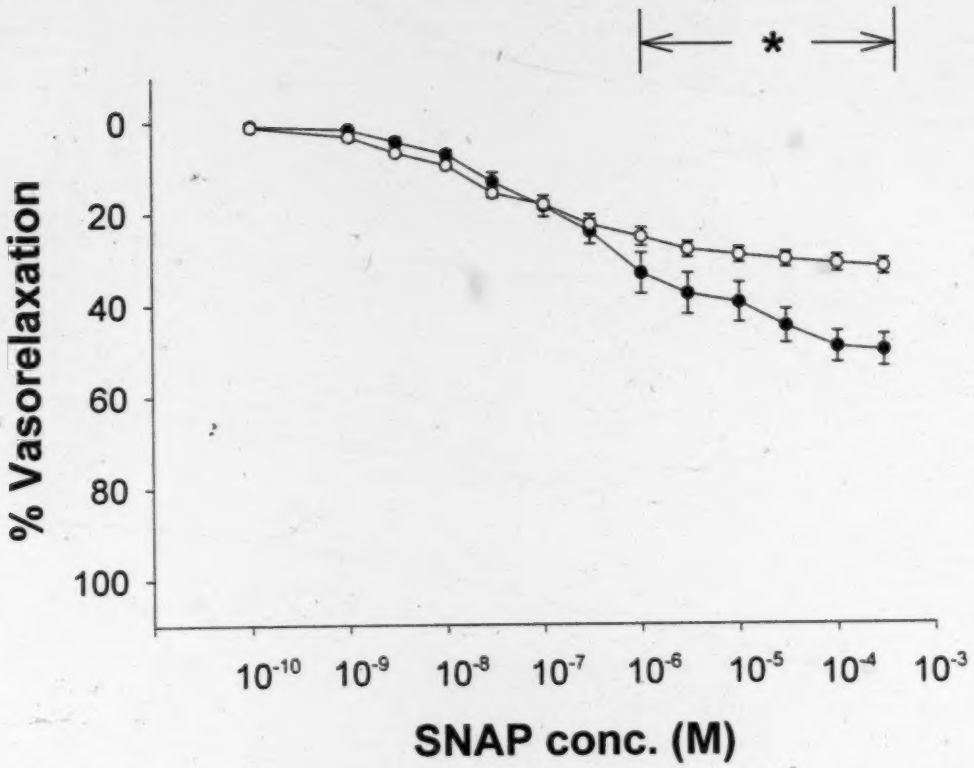

Figure 28. Vasorelaxant effects of s-nitroso-n-acetyl-D, L-penicillamine (SNAP) on hilar arteries (closed circles, n=11) and veins (open circles, n=8). Cumulative dose-response curves to SNAP, following preconstriction with phenylephrine (EC_{80}). Vertical bars delineate standard error of the mean. *, Significant difference between splenic resistance artery and vein ($p<0.05$). Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

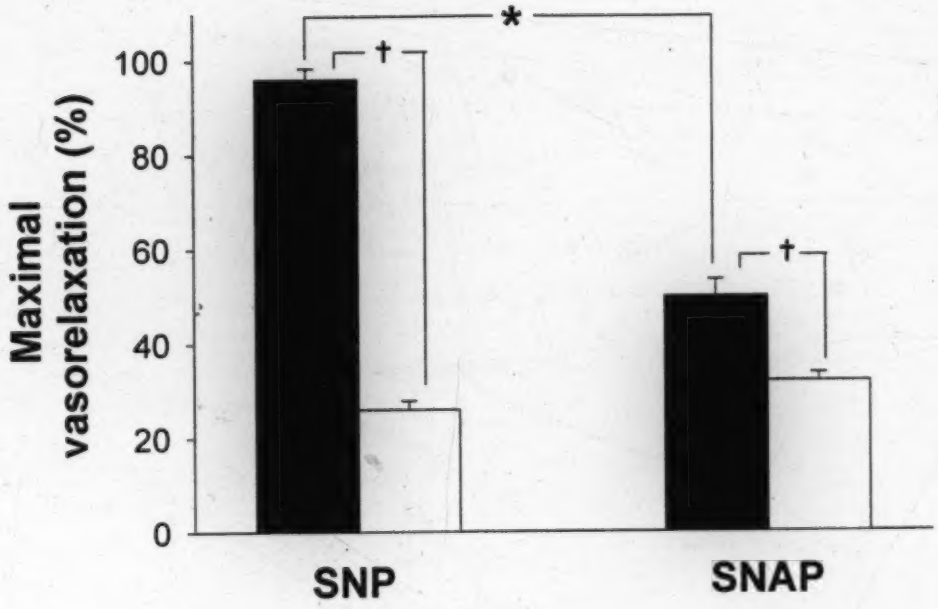

Figure 29. Maximal vasorelaxation to sodium nitroprusside (SNP) and S-nitroso-N-acetyl-D,L-penicillamine (SNAP) in hilar arteries (closed bars; n=9 and n=11, respectively) and veins (open bars; n=10 and n=8, respectively). Vertical bars delineate standard error of the mean. *, Significant difference in maximal vasorelaxation of hilar arteries to SNP and SNAP. †, Significant difference between responses of hilar vessels to SNP and SNAP. One-way ANOVA on ranks plus Dunn's test for multiple comparisons.

There was a dose-dependent decrease in tension (vasorelaxation) in phenylephrine-preconstricted vessels in response to both SNP (Fig. 27) and SNAP (Fig. 28). However, the maximum vasorelaxation to SNP was significantly greater in arteries (96 $\pm$ 2%, n=9) than veins (26 $\pm$ 2%, n=10) ($p<0.05$) (Fig. 27, 29) i.e. SNP was fully able to counteract the constrictive activity of phenylephrine in arteries, but not in veins. Similarly, the maximum vasorelaxation to SNAP was significantly greater in arteries (50 $\pm$ 4%, n=11) than veins (32 $\pm$ 2%, n=8) ($p<0.05$) (Fig. 28, 29). In splenic arteries, the maximum vasorelaxation to SNP (96 $\pm$ 2%, n=9) was significantly greater than to SNAP (50 $\pm$ 4%, n=11) (*$p<0.05$) (Fig. 29). By contrast, in veins, there was no significant difference between the maximal vasorelaxation to SNP and SNAP ($p>0.05$) (Fig. 29).

EXPERIMENT F: EFFECT OF N^G-MONOMETHYL-L-ARGININE (L-NMMA) ON ADRENOMEDULLIN (ADM)-INDUCED RELAXATION OF ISOLATED NORMAL SPLENIC RESISTANCE ARTERIES.

Neither L- nor D-NMMA caused a significant change in wall tension of phenylephrine preconstricted hilar arteries (ADM + L-NMMA: 0.02 $\pm$ 0.01 mN/mm, n=3 versus ADM + D-NMMA: 0.02 $\pm$ 0.01 mN/mm, n=3) ($p<0.05$). L-NMMA treated hilar arteries, but not D-NMMA treated hilar arteries, significantly inhibited the ADM-induced relaxation (maximal relaxation ADM + L-NMMA: 38 $\pm$ 3%, n=5 versus ADM + D-NMMA: 60 $\pm$ 3%, n=5) ($p<0.05$) (Fig. 30).

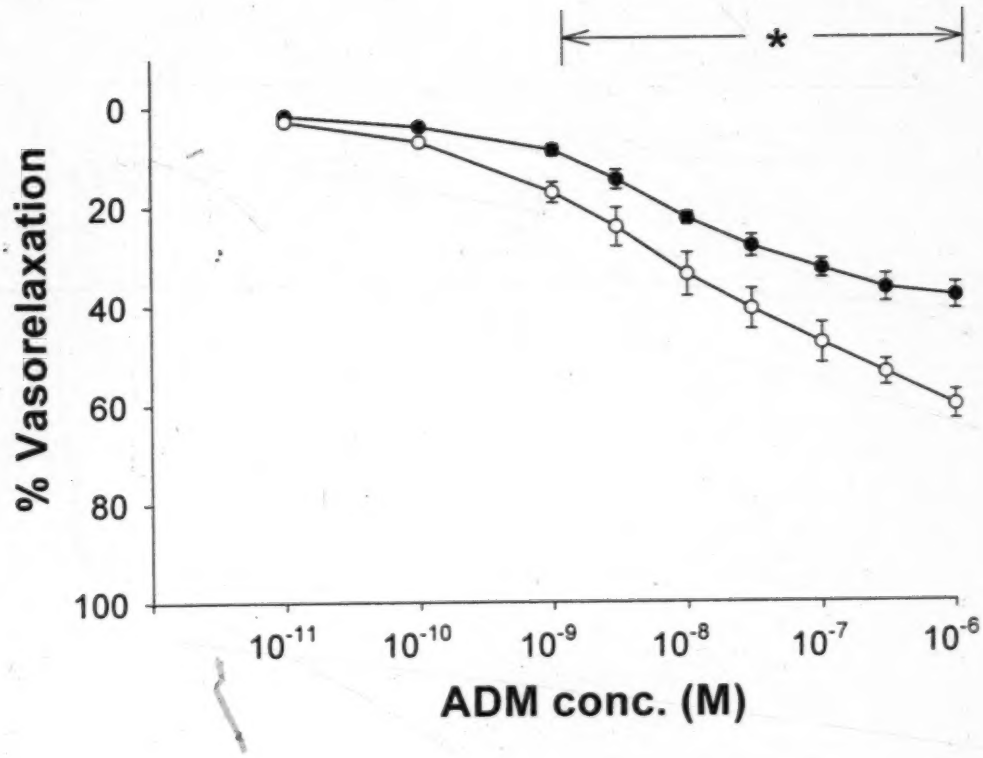

Figure 30. Effect of nitric oxide (NO) synthase inhibition on adrenomedullin (ADM)-induced vasorelaxation of splenic resistance arteries. Vessels treated with ADM plus L-NMMA (closed circles, n=5) or with ADM plus D-NMMA (open circles, n=5). The vessels were preconstricted with phenylephrine (EC_{80}). *, Significant difference between % vasorelaxation, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

EXPERIMENT G: EFFECT OF PHENYLEPHRINE, ENDOTHELIN-1 (ET-1), S-NITROSO-N-ACETYL-D,L-PENICILLAMINE (SNAP) AND ADRENOMEDULLIN (ADM) ON VASOREACTIVITY OF ISOLATED LIPOPOLYSACCHARIDE (LPS)-EXPOSED (150μg/kg/hr for 3 hr *in vivo*) SPLENIC RESISTANCE ARTERIES AND VEINS.

There was a significant concentration-dependent increase in tension with phenylephrine ($1x10^{-8}$ to $1x10^{-3}$ M) in both 3 hr LPS-exposed hilar arteries (n=13) and hilar veins (n=9) ($p<0.05$). There was no significant difference in the phenylephrine EC_{80} values between 3 hr LPS-exposed hilar arteries (6.1×10^{-6} M $\pm$ 1.0×10^{-6} M) and veins (6.4×10^{-6} M $\pm$ 1.1×10^{-6} M) (Fig. 45; Appendix J) ($p<0.05$) i.e. although the individual pre-constrictive EC_{80} doses used for vessels varied slightly, the mean EC_{80} dose of phenylephrine applied to arteries was not significantly different from that used for veins. Nor was there any significant difference in the phenylephrine EC_{80} values between 3 hr LPS-exposed hilar arteries and veins and their respective normal hilar arteries and veins (experiment E) (Fig. 45; Appendix J) ($p>0.05$).

ET-1 caused a dose-dependent vasoconstriction of both splenic resistance arteries and veins ($p<0.05$) (Fig. 31). The potency of ET-1 was greater on the veins than the arteries (EC_{50}= artery 3.0×10^{-9} M $\pm$ 3.9×10^{-10} M, n=13 versus vein 6.0×10^{-10} M $\pm$ 5.9×10^{-10} M, n=8). There was no significant difference in the ET-1 EC_{50} values between 3 hr LPS-exposed hilar arteries and veins and their respective normal hilar arteries and veins (experiment E) (Fig. 46; Appendix K) ($p<0.05$).

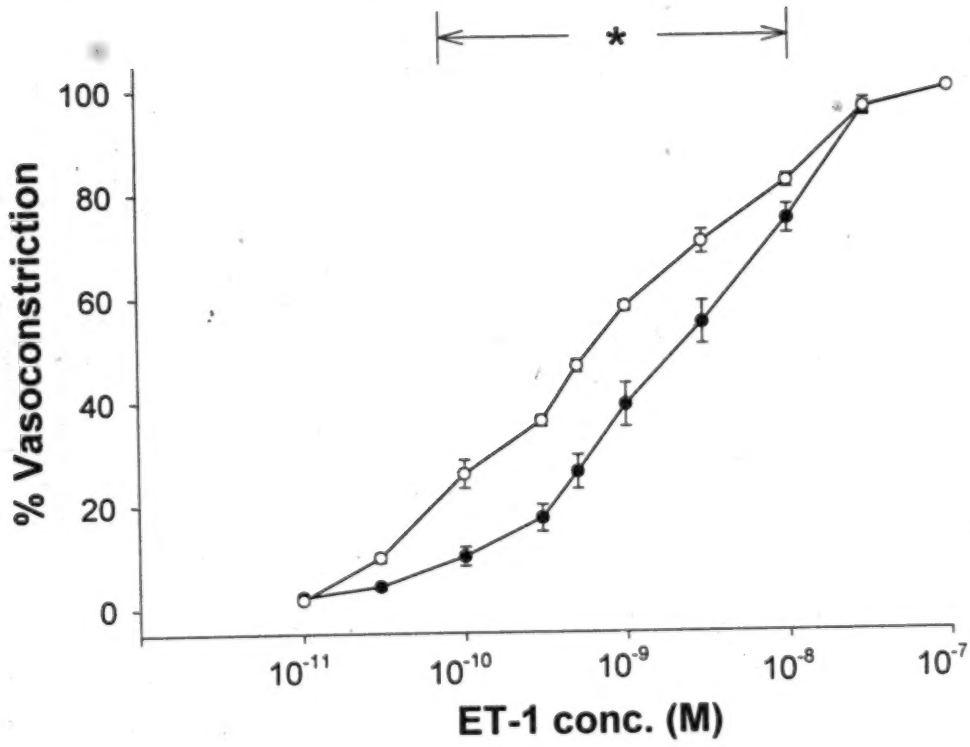

Figure 31. Vasoconstrictor effect of endothelin-1 (ET-1) on 3 hr lipopolysaccharide (LPS)-exposed splenic resistance arteries (closed circles, n=13) and veins (open circles, n=8). *, Significant difference between % vasoconstriction of artery and vein, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

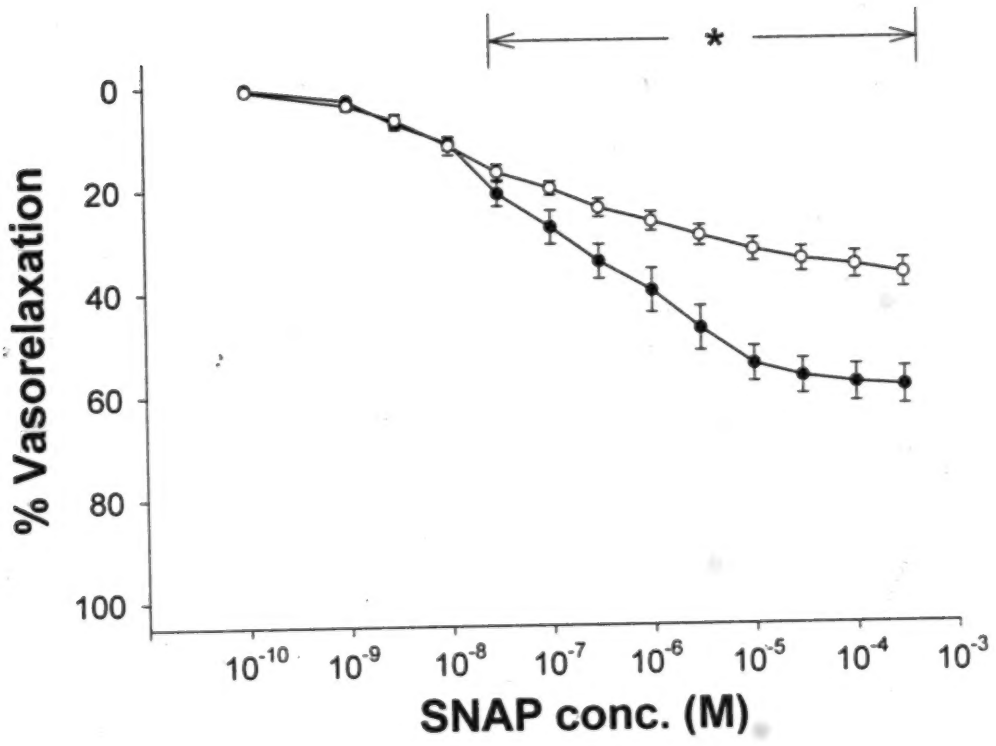

Figure 32. Vasorelaxant effects of s-nitroso-n-acetyl-D, L-penicillamine (SNAP) on 3 hr lipopolysaccharide (LPS)-exposed hilar arteries (closed circles, n=12) and veins (open circles, n=11). Cumulative dose-response curves to SNAP, following preconstriction with phenylephrine (EC_{80}). Vertical bars delineate standard error of the mean. *, Significant difference between splenic resistance artery and vein ($p<0.05$). Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

There was a dose-dependent decrease in tension (vasorelaxation) in 3 hr LPS-exposed phenylephrine-preconstricted vessels in response to SNAP ($p<0.05$) (Fig. 32). The maximum vasorelaxation to SNAP was significantly greater in arteries ($59 \pm 4\%$, n=12) than veins ($37 \pm 3\%$, n=11) ($p<0.05$). There was no significant difference in the maximal vasorelaxation to SNAP between 3 hr LPS-exposed hilar arteries and veins and their respective normal hilar arteries and veins (experiment E) (Fig. 47; Appendix L) ($p>0.05$).

There was a dose-dependent decrease in tension (vasorelaxation) in 3 hr LPS-exposed phenylephrine-preconstricted vessels in response to ADM ($p<0.05$) (Fig. 33). The maximum vasorelaxation to ADM was significantly greater in arteries ($63 \pm 8\%$, n=11) than veins ($43 \pm 7\%$, n=9) ($p<0.05$). There was no significant difference in the maximal vasorelaxation to ADM between 3 hr LPS-exposed hilar arteries and veins and their respective normal hilar arteries and veins (as described in experiment E) (Fig. 48; Appendix M) ($p>0.05$).

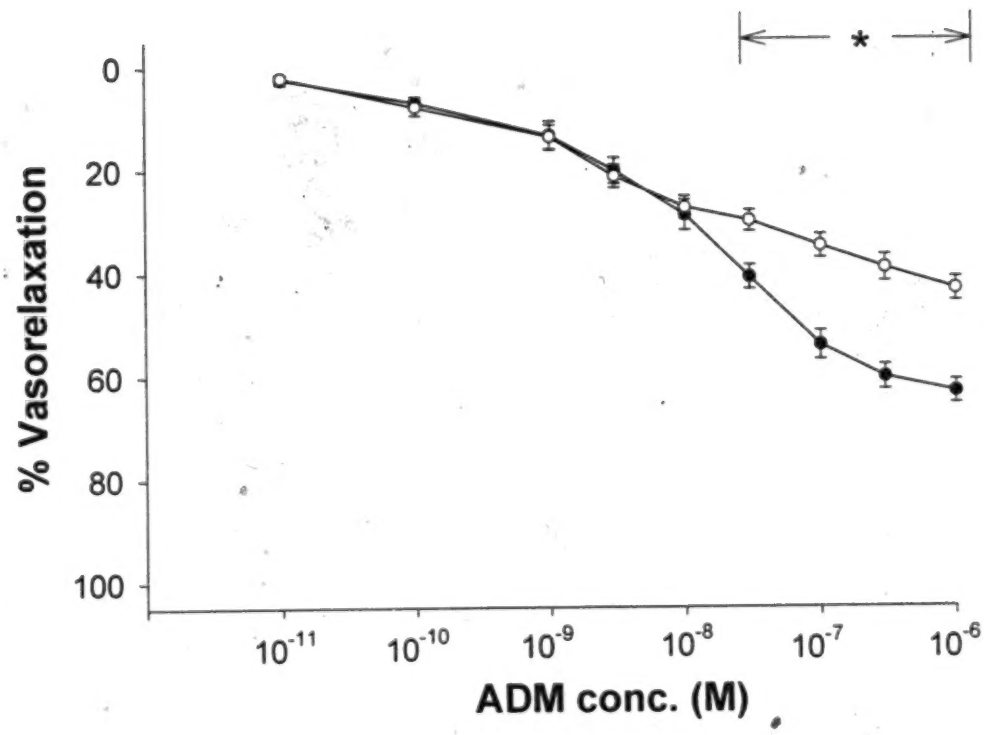

Figure 33. Effect of adrenomedullin (ADM) on vasorelaxation of 3 hr lipopolysaccharide (LPS)-exposed splenic resistance arteries (closed circles, n=11) and veins (open circles, n=9). The vessels were preconstricted with phenylephrine (EC_{80}). *, Significant difference between % vasorelaxation of artery and vein, $p<0.05$. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

CHAPTER 4.

DISCUSSION

EXPERIMENT A: EFFECT OF SPLENECTOMY ON LIPOPOLYSACCHARIDE (LPS)-INDUCED HYPOTENSION, HEMOCONCENTRATION AND HYPOVOLEMIA IN CONSCIOUS MALE RATS.

These *in vivo* experiments were designed to investigate the importance of the spleen in the early hemodynamic perturbations in blood pressure and plasma volume during endotoxemia. In addition, measurements of urine output and plasma protein concentration over the period of LPS infusion were taken in order to determine if similarities existed between our rat model of endotoxemia and clinical septic shock. We reasoned that, if enhanced fluid extravasation from the splenic circulation into the lymphatic system does indeed contribute to a loss of cell-free fluid from the blood following intravenous infusion of LPS, then splenectomy should reduce or prevent the early perturbations in blood pressure and plasma volume during endotoxemia. This is exactly what was found.

In the intact rats, MAP fell from 101 $\pm$ 2 mmHg to 88 $\pm$ 4 mmHg (n=7), and then stabilized at about 90 mmHg (Fig. 13). Hematocrit rose from 41 $\pm$ 1% to 44 $\pm$ 1% at 90 min (Fig. 14), at which time there was tachycardia present (Fig. 15), and plasma volume had fallen from 4.7 $\pm$ 0.12 ml/100g body wt. to 4.0 $\pm$ 0.05 ml/100g body wt. (Fig. 16). In the splenectomized rats MAP did not fall, neither did hematocrit rise, and a significant tachycardia was present only in the latter hours of LPS infusion. Nor was there any change in plasma volume i.e. splenectomy prevented the hypotension, hemoconcentration and hypovolemia customarily induced by LPS. Splenic arterial and venous blood flows measured simultaneously in conscious intact rats infused with LPS indicated an LPS-induced increase in intrasplenic fluid efflux (experiment B) (Fig. 19C). There is no

denying that ultimately, LPS causes a generalized increase in capillary permeability (52,113,241). However, we have specifically examined the mechanisms underlying the *early* (specifically within the intial 3 hr period of endotoxemia) hemodynamic perturbations following i.v. infusion of LPS, before there is widespread endothelial damage. Indeed, our measurements of plasma protein concentration (Fig. 17) suggest that increased capillary permeability occurs during the latter hours of LPS infusion (by 3 hr and beyond) in our rat model of endotoxemia. However, early endotoxin-induced changes in plasma volume (at 90 min post-LPS infusion) (Fig. 16) precede the loss of integrity of the capillary wall (indicated by a reduced plasma protein concentration at approximately 3 hr post-LPS infusion onwards) (Fig. 17). Furthermore, during this early period (considered to be between 0-3 hr of LPS infusion), plasma protein concentration did not significantly change despite the hypotension (Fig. 13), hemoconcentration (Fig. 14) and fall in plasma volume (Fig. 16).

Experiment B (Fig. 19C) shows that the infusion of LPS induced an average increase in splenic A-V flow difference of approximately 0.5 ml/min above baseline values in conscious rats. If this rate of fluid efflux occurred in experiment A, it can be estimated that during the initial 90 min period of LPS infusion in intact conscious rats, extravasation from the splenic circulation would have increased by about 45 ml. Normally, this extravasated fluid is continuously returned from the lymphatic system to the blood (119). During endotoxemia, the circulation of lymph is dysfunctional (70,94,128) and the capacity of the lymphatic system is increased by such agents as ANF (186), the circulating levels of which rise in endotoxic shock (5). This would allow for

storage of the approximately 3.5 ml that disappeared from the circulation after infusion of LPS for 90 min (Fig. 16). Considering that the blood volume of these rats is only about 35 ml (18), it is apparent that intrasplenic fluid efflux could contribute to the hypotension, hemoconcentration and hypovolemia observed after administration of LPS (Figs. 13, 14, 16).

It should be pointed out that, in contrast to our studies and those of Gardiner et al (1994) (88), many researchers in this field have used acute (bolus) administration of very much higher doses of endotoxin (25,171,176), or have not recorded hemodynamic data during the early stages on endotoxemia (81). Our rat model of endotoxemia possessed pathophysiological features, namely hypotension (Fig. 13), hemoconcentration (Fig. 14), tachycardia (Fig. 15), hypovolemia (Fig. 16), hypoproteinemia (Fig. 17), and oliguria (Fig. 18) that are consistent with the changes that have been reported to occur in septic shock (14,190,217). Although we may conclude that the systemic load of LPS achieved in our study was probably no greater than those found in animals subjected to CLP, the differences in the distribution of that load, and of the mechanisms underlying the development of cardiovascular derangement and circulatory shock, make it difficult to compare the changes in plasma volume obtained in experiment A with those obtained using CLP (249) where blood volume was reported to be reduced only after 20 hr.

Two particular questions arise from the initial results of experiment A. First, what is the mechanism of this intrasplenic fluid extravasation and second, why should the spleen respond in this way to endotoxemia? The increase in fluid efflux within the splenic

circulation does not appear to involve changes in capillary permeability, given the discontinuous endothelium that lines the splenic vasculature (232) and the fact that the splenic vascular system is normally freely permeable to albumin (46). Hence, the control must lie at pre-capillary sphincters controlling the route that blood takes once it enters the spleen. Under basal conditions, a large proportion of the splenic blood flow is shunted through A-V anastomoses, the so-called "fast route" (102). By altering the tone of selective sphincters, blood can be directed through the "slow route". We propose that, by altering the pre-/post-capillary resistance, changes can be brought about in the capillary P_C within the spleen, in much the same way as afferent/efferent arteriolar vasoconstriction or dilatation controls glomerular filtration rate in the kidney. As a consequence of this alteration in splenic capillary P_C, fluid moves from the blood into the extravascular space i.e. into the splenic lymphatic system.

The reason for this fluid extravasation may lie with the nature of the body's response to pathogenic insult. T-cells will be activated and released into the circulation. The major site of such activity is the spleen; the activated T-cells are disseminated, not by release directly into the venous outflow from the spleen, but through lymphatic drainage (32,124,193,211). An increase in lymph flow thus facilitates the immune response (43). Unfortunately, this fluid derives from the plasma. As a result, if the increase in lymph flow is very large, there will be a significant reduction in plasma volume. Loss of plasma volume contributes to the hemodynamic perturbations in MAP (Fig. 13) despite the activation of reflex and hormonal cardiovascular mechanisms that attempt to maintain a

physiologically optimal MAP (Figs. 4, 7, 8). We propose that this balance of fighting the infection and preserving the integrity of the circulation is perturbed in septic shock (7).

There are studies that suggest that splenectomy (which removes the pathway for intrasplenic fluid efflux from the intravascular compartment into the systemic lymphatic system) attenuates LPS-induced hemodynamic changes in dogs (51,63). In brief, these studies reported that splenectomized dogs were less prone than intact dogs to developing a characteristic hypotension and hemoconcentration following i.v. infusion of LPS. Therefore, we suggest that, the significance of intrasplenic fluid efflux in the regulation of plasma volume and MAP during endotoxemia is not peculiar to the rat. The results of experiment A indicate that the spleen may be crucially involved in the hemodynamic perturbations during the initial stages of endotoxemia (and perhaps beyond). Because of the dual roles of the spleen (immunologic and cardiovascular), together with the obvious ethical and clinical considerations, it would not be desirable to splenectomize patients in septic shock. However, an understanding of what controls intrasplenic fluid efflux could be beneficial. Therefore, neural control of this fluid efflux within the splenic circulation was addressed in experiment B.

EXPERIMENT B: EFFECT OF SPLENIC DENERVATION ON LIPOPOLYSACCHARIDE (LPS)-INDUCED HYPOTENSION, HEMOCONCENTRATION AND HYPOVOLEMIA IN CONSCIOUS MALE RATS.

To our knowledge, there are no previously published studies concerning the effects of splenic denervation on the cardiovascular response to endotoxin in the rat or any other

species. This series of experiments sought to investigate the influence of the splenic nerves on the changes in intrasplenic fluid efflux and hemodynamic perturbations following infusion of LPS in conscious rats. Splenic denervation (verified by comparison of the total catecholamine content of intact and denervated splenic tissue) removes sympathetic vasoconstrictor tone, predominantly over pre-capillary splenic resistance vessels (3,22,180). This has two effects. First, splenic denervation impairs the contribution of the splenic vasculature to the reflex increase in TPR in response to the LPS-induced decrease in MAP (Fig. 20A). Second, splenic denervation prevents the LPS-induced increase in SSNA from limiting a rise in intrasplenic P_C (which drives intrasplenic fluid efflux) (157,228,229). Consequently, splenic denervation had been expected to exaggerate intrasplenic fluid efflux and thus worsen the hypotension, hemoconcentration, and hypovolemia following infusion of LPS. This is exactly what was found (Figs. 19, 20, 22).

Under euvolemic conditions in conscious rats, as much as 25 % of fluid flowing into the spleen is removed from the circulating blood (46). This is consistent with the splenic A-V flow differential of ~0.9 ml/min at time=0 (Fig. 19C) that occurs in both intact and denervated groups. In intact rats, infusion of LPS caused a significant increase in splenic A-V flow differential (Fig. 21), indicating enhanced intrasplenic fluid efflux into extravascular spaces. This was exacerbated in splenic denervated rats. The LPS-induced increase in intrasplenic fluid efflux was associated with hypotension (Fig. 20A), hemoconcentration (Fig. 20B), tachycardia (Fig. 21D) and hypovolemia (Fig. 22). Specifically, the early LPS-induced hypotension (Fig. 21B) was consistent with an

increase in splenic A-V flow differential (Fig. 21A), in both intact and denervated rats. Moreover, the LPS-induced hemodynamic perturbations were exacerbated in splenic denervated rats. Our finding that there were no significant differences in splenic tissue wet weight between intact and splenic denervated rats infused with LPS, despite significant differences in the splenic A-V flow differential, is consistent with the report that the rat spleen is non-compliant and cannot acutely store blood volume (199).

The time course of differences in intrasplenic fluid efflux and decreases in MAP, between intact and denervated rats infused with LPS, does not suggest a simple causal relationship. First, the more pronounced fall in MAP in the denervated rats (at 20 min and 40 min post-LPS infusion) precedes the differences in intrasplenic fluid efflux (Figs. 21A, 21B). But, shortly thereafter, the time course of differences in intrasplenic fluid efflux and decreases in MAP are correlated (at 60 min, 3 hr and 8 hr post-LPS infusion) (Figs. 19, 20A). The transient recovery of MAP in LPS-infused denervated rats (at 90 min) is correlated with an exaggerated increase in heart rate (Figs. 21B, 21D). We suggest that, the exaggerated hypotension may be driving the enhanced reflex tachycardia in denervated rats infused with LPS. At the time of significant increases in hematocrit and intrasplenic fluid efflux (90 min post-LPS infusion) (Figs. 21A, 21C), plasma volume in the denervated rats was significantly less than in the intact rats (Fig. 22). We propose that the reduced plasma volume in denervated rats compared to intact rats at 90 min post-LPS infusion (Fig. 22) was due to the exaggerated intrasplenic fluid efflux that occurred in the preceding time period in denervated as opposed to intact rats i.e. at 60 to 90 min post-LPS infusion (Fig. 21A). There was a significant time-dependent increase in PRA in

intact and denervated LPS-infused groups (Fig. 23). The exaggerated increase in PRA in the denervated rats is consistent with the greater fall in plasma volume. Although an exaggerated intrasplenic fluid efflux may not account entirely for the changes in MAP following LPS infusion into the bloodstream, our results do provide evidence that enhanced fluid extravasation from the splenic circulation may contribute to the exaggerated LPS-induced hypotension, hemoconcentration and hypovolemia in the early hours of endotoxemia in denervated rats. These results thus support the proposal that SSNA limits intrasplenic fluid efflux during endotoxemia.

We estimated lymphatic flow from the splenic circulation by measuring the difference between splenic arterial inflow and venous outflow (Fig. 19C). Although a direct measure of lymphatic flow from the spleen would have been preferred, this is not technically feasible in the rat, given that there are several branches of the splenic lymph duct that cannot be placed together in a blood flow probe without damaging them. However, vascular isolation of the spleen was ensured. Therefore, by the law of mass action, the difference between the volume of blood going into the spleen and the volume coming out must represent the fluid volume leaving the splenic circulation. Since the weight of the spleen did not change despite significant changes in splenic A-V flow differential (Fig. 19C), and since the volume of extravasate was many times the total volume capacity of the rat spleen, this extravasated fluid must have been transferred to another site, namely the systemic lymphatic system. The reported accuracy of the transonic flow probes is $\pm$ 2 % (Transonic Systems Inc., Ithaca, NY, USA), which relates to a detectable difference in A-V blood flows of ~0.05 ml/min. Hence, given such confidence in our reported values

for splenic blood flow, the mean increase in splenic A-V flow difference between intact (control –0.01 ± 0.02 ml/min versus LPS +0.21 ± 0.06 ml/min) and denervated (control – 0.03 ± 0.01 ml/min versus LPS +0.41 ± 0.08 ml/min) rats must be viewed with significance.

The fact that plasma volume was less in denervated than intact rats at 90 min post-LPS infusion (Fig. 22) is probably a combination of enhanced intrasplenic fluid efflux plus increased capacity of the lymphatic system to hold onto this extravasated fluid. The increase in PRA plus the tachycardia, in both intact and denervated LPS-infused groups, was anticipated considering that plasma volume contraction is a major stimulus for both increased PRA (41) and for tachycardia (26,117). The greater increase in PRA and the exaggerated increase in heart rate in the denervated compared to the intact LPS-infused group is consistent with the exaggerated loss of plasma volume in the denervated animals (Figs. 21D, 22).

Another consequence of splenic denervation, apart from the removal of sympathetic vasoconstrictor innervation of the splenic vasculature (3,22,180), would be removal of the sensory afferent innervation of the spleen. These sensory afferents have been proposed to form part of a neural reflex pathway between the spleen and kidney termed a spinal splenorenal reflex (112,164,165,252). The sensory afferent traffic from the spleen inhibits RSNA, thus limiting PRA. Removal of this inhibition (by splenic denervation) would consequently elevate RSNA, and thus eventually cause a rise in plasma volume over the long term i.e. over the one week period of recovery after surgery. The results in

experiment B lend credence to this proposal given that splenic denervated rats had increased basal PRA plus an expanded plasma volume compared to intact rats (Figs. 22, 23).

In interpreting our findings, it is important to distinguish between short-term (minutes to hours) and long-term (days) regulation of plasma volume and MAP. The long-term regulation is more likely to involve renal control of extracellular fluid volume. It is an alteration in these mechanisms that would be responsible for increasing *basal* plasma volume in the splenic denervated rats (Fig. 22). PRA in the splenic denervated, saline-infused control animals was elevated in comparison to the intact saline-infused controls (Fig. 23). The rise in PRA in denervated rats may be the primary driving force behind the elevated basal plasma volume observed in this series of experiments.

Experiment B showed an increase in splenic arterial blood flow following intravenous infusion of LPS (Fig. 19A); this has also been reported elsewhere (203). The increased splenic perfusion during endotoxemia (203) is perhaps not surprising given that the spleen is the primary lymphoid organ and comprises a large number of immune cells that need to migrate into the bloodstream in order to combat the pathogenic insult. Thus, the hemodynamic consequences of septic shock in the splanchnic circulation may involve a redistribution of blood flow to organs such as the spleen, which is necessary for host defense mechanisms (Fig. 2). Although there is no evidence directly pertaining to autoregulation within splenic vessels, there are reports of autoregulatory mechanisms within the hepatic arterial/portal venous and mesenteric vascular beds (97) (Fig. 2). It

may be speculated that the splenic circulation possesses strong autoregulatory mechanisms that help maintain a high rate of blood flow to the spleen, in order to ensure that splenic function is maintained.

As well as directly affecting the vascular tone of splenic resistance arteries, the splenic sympathetic nerve has been suggested to mediate central nervous system modulation of splenic immune cell function (116). Activation of immune cells elevates their production of vasoactive cytokines (91,132,163,166,212). Thus, sympathetic nerve activity can modulate the secretion of cytokines from LPS-exposed immune cells (158), thereby altering the production of vasoactive agents. Hence, denervation of the spleen may remove the inhibitory action of sympathetic nerves on the production of vasoactive cytokines by splenic immune cells. This alteration in production of vasoactive cytokines could potentially have contributed to the exaggerated hemodynamic perturbations found in the splenic denervated rats following infusion of LPS. However, our measurements of plasma TNF-α concentration do not support this proposal. TNF-α is one of the first primary cytokines released during sepsis (29) and it has been implicated in the LPS-induced hemodynamic changes (89,90,175,247). In experiment B, the plasma TNF-α concentration increased equally in the denervated and intact rats in response to LPS i.e. splenic denervation did not significantly attenuate the early LPS-induced increase in plasma TNF-α levels ($p>0.05$). Thus, altered production of vasoactive cytokines cannot account for the LPS-induced hemodynamic differences we have observed between intact and denervated rats.

The results from experiment B confirm the importance of the splenic nerves in influencing plasma volume and MAP homeostasis during endotoxemia. Splenic denervation enhances intrasplenic fluid efflux, and worsens LPS-induced hypotension, hemoconcentration and hypovolemia (9). We propose that in intact animals, SSNA limits intrasplenic fluid extravasation by opposing the activity of endotoxin-induced vasodilatory factors. Splenic denervation unmasks the full vasodilatory activity of these factors, thus increasing intrasplenic P_C , and subsequently exacerbating fluid extravasation from the intravascular compartment into the systemic lymphatic system. The consequence is an exaggerated LPS-induced loss in plasma volume and reduction in MAP. The hormonal control of intrasplenic fluid efflux is the topic of concern in the discussion of experiment C.

EXPERIMENT C: EFFECT OF INTRASPLENIC INFUSION OF ADRENOMEDULLIN (ADM) AND S-NITROSO-N-ACETYL-D,L-PENICILLAMINE (SNAP) ON SPLENIC ARTERIAL VERSUS VENOUS HEMATOCRIT AND WET SPLENIC TISSUE WEIGHT IN ANESTHETIZED MALE RATS.

Intrasplenic infusion of ADM and SNAP caused a significant increase in splenic venous hematocrit compared to arterial hematocrit ($p<0.05$), but no change in wet splenic tissue weight ($p>0.05$) i.e. ADM and SNAP increased the loss of cell-free fluid from the splenic circulation into the systemic lymphatic system. Since the measured wet splenic tissue weight did not change and given evidence that the volume of translocated fluid from the

splenic circulation is many times larger than the total volume capacity of the spleen, the extravasated fluid from the splenic circulation cannot have been accommodated within the parenchyma of the spleen. Although NO (specifically derived from iNOS) has been suggested to increase capillary permeability (78), the importance of this effect in the splenic microcirculation would be minimal considering that the splenic vascular endothelium is discontinuous (232) and so readily allows the passage of protein-rich fluid from the intravascular compartment into extravascular spaces (46,135). Therefore, the ADM- and SNAP-induced increase in splenic A-V flow differential that has been observed (Kaufman, unpublished observation) (8), together with the results of experiment C, must represent lymphatic drainage.

During endotoxemia, where there is increased biosynthesis of ADM and NO (73,93,183,218), the actions of ADM and NO on splenic resistance vessels may contribute to the increased intrasplenic fluid efflux following i.v. infusion of LPS (experiment B: Figs. 19C, 21A). The effects of intrasplenic infusion of ADM and SNAP on splenic arterial versus venous hematocrit could be a consequence of differential vasoreactivity of splenic resistance vessels to these vasoactive factors (experiment E).

EXPERIMENT D: EFFECT OF ADRENOMEDULLIN (ADM) ON INTRASPLENIC MICROVASCULAR PRESSURE.

We hypothesized that ADM contributes to intrasplenic fluid extravasation by inducing greater vasorelaxation in splenic resistance arteries than veins, such that intrasplenic P_C

rises. In the isolated, blood-perfused spleen, intrasplenic infusion of ADM into an anesthetized rat caused an increase in intrasplenic P_C ($p<0.05$), due to a fall in pre-capillary resistance ($p<0.05$); there was no change in post-capillary resistance ($p>0.05$).

Intrasplenic infusion of SNAP into an anesthetized rat has been reported to cause a significant increase in intrasplenic P_C (8). This is relevant to experiment D, since ADM-induced vasorelaxation is partly mediated by NO (110) (experiment F) (Fig. 29). Moreover, there is evidence for differential vasoreactivity between splenic resistance arteries and veins to ADM (Kaufman, unpublished observation) (experiments E and G) (Figs. 24, 33), which we propose is the mechanism causing the ADM-induced increase in intrasplenic P_C. It may be calculated that, at a splenic blood flow (perfusion rate) of 1 ml/min, the plasma concentration of ADM that was created was approximately 10^{-8} M. At this concentration *in vitro*, ADM-induced vasodilation is significantly greater in the splenic resistance arteries than in the veins (Figs. 24, 33).

EXPERIMENT E: EFFECT OF ADRENOMEDULLIN (ADM), ATRIAL NATRIURETIC FACTOR (ANF), ENDOTHELIN-1 (ET-1), SODIUM NITROPRUSSIDE (SNP), AND S-NITROSO-N-ACETYL-D,L-PENICILLAMINE (SNAP) ON VASOREACTIVITY OF ISOLATED NORMAL SPLENIC RESISTANCE ARTERIES AND VEINS.

We proposed that there would be a differential vasorelaxant response between splenic hilar arteries and veins to the vasoactive agents ADM, ANF, ET-1 and NO. The results

validated our hypothesis. ANF and ET-1 caused a dose-dependent vasoconstriction of both splenic resistance arteries and veins (Figs. 25, 26) ($p<0.05$). The potency of ANF and ET-1 was greater on the veins than the arteries (Figs. 25, 26) ($p<0.05$). Vasoreactivity of preconstricted splenic resistance vessels to ADM, SNP and SNAP indicated a significantly greater relaxation of arterioles than of venules (Figs. 24, 27, 28). These results are consistent with our proposal that differential vasoreactivity of splenic resistance arteries and veins to ADM, ANF, ET-1 and NO elevates intrasplenic P_C. The results provide evidence for the mechanism causing the ANF-induced increase in intrasplenic P_C (228), the ADM-induced increase in intrasplenic P_C (experiment D) (Kaufman, unpublished observation), and the SNAP-induced increase in intrasplenic P_C (8).

It may be calculated that, at a splenic blood flow of 2.5 ml/min in the intact anesthetized rat (8) and with a SNAP infusion rate of 0.3μg/10μl/min, the splenic arterial plasma concentration of SNAP that caused increased intrasplenic fluid efflux was approximately $0.6x10^{-6}$ M. At this concentration in experiment E, the cumulative dose-response curves for hilar artery and vein diverge and reveal a significant difference in vasoreactivity to SNAP (Fig. 28). SNAP also had greater efficacy (maximal vasorelaxation) on hilar arteries than veins (Fig. 29). Thus, at the doses used in the Andrew et al study (8) that caused increased intrasplenic fluid efflux, the *in vitro* results of experiment E suggest that NO caused a greater vasorelaxation of the splenic resistance arteries than of the veins.

The differential response of splenic resistance arteries and veins to ADM- and NO-induced vasorelaxation may be due to a number of reasons. There may be variations between splenic resistance arteries and veins in their reliance upon NO as a vasodilator mechanism (12,55). This has been suggested to explain the divergent responses (both quantitative and qualitative) of different vessels within a single vascular bed to a common stimulus, specifically in regard to NO-induced vasorelaxation (12,55). Studies have reported that NO donors cause greater vasorelaxation of veins than arteries (86). Our results show the opposite, which may indicate that the response of the splenic vasculature is distinct and unique from that of the general circulation. Berthiaume et al (1995) reported that ADM induced a selective arterial vasodilation in a double-perfused mesenteric bed preparation of the rat, which is consistent with our findings in the spleen (27). Further possible reasons for the differential responses between splenic resistance arteries and veins to NO-induced vasorelaxation may reside in differences in the rate of NO generation at the cell surface of venous VSMCs versus arterial VSMCs, differences in cellular entry mechanisms for NO into VSMCs, and variation in sensitivity of different soluble guanylate cyclases in venous VSMCs versus arterial VSMCs (159).

Despite their biochemical differences (184), SNP and SNAP are both termed nitrovasodilators. These are vasorelaxants that act via the formation of NO and its subsequent activation of VSMC soluble guanylate cyclase (177). Our use of both SNP and SNAP as endothelium-independent NO donors was based on evidence of different NO redox species donated by these agents (75,222) and the possible influence this may have on NO-induced vasorelaxation in isolated vessels (86,159). The results suggest that

the efficacy (maximal relaxation) of SNAP and SNP on splenic resistance arteries (but not veins) is different (Fig. 29). The biochemical differences between SNAP and SNP may be reasoned to cause the variation in vasorelaxation achieved with each NO donor. Under physiological conditions, NO can interconvert between the redox forms of NO^-, $NO^\cdot$, and NO^+ (40,222). This redox versatility has impact on NO-induced vasorelaxation and, although controversial (13), it is suggested that guanylate cyclase activation has preference for the $NO^\cdot$ redox form (67). Since SNP releases less $NO^\cdot$ redox form than SNAP (76), a greater vasorelaxant response might be expected in vessels exposed to SNAP than SNP. However, we did not find this; there was significantly greater vasorelaxation with SNP than SNAP on splenic resistance arteries, but no significant difference in maximal vasorelaxation to either nitrovasodilator on veins (Fig. 29). The basis for these responses remains unclear.

EXPERIMENT F: EFFECT OF N^G-MONOMETHYL-L-ARGININE (L-NMMA) ON ADRENOMEDULLIN (ADM)-INDUCED RELAXATION OF NORMAL SPLENIC RESISTANCE ARTERIES.

Our results show that ADM-induced vasorelaxation of splenic resistance arteries is attenuated by L-NMMA-mediated inhibition of NO synthase activity (Fig. 30). Whether splenic resistance veins behave in a similar manner is unknown. The source of this NO (endothelial or VSMCs) was also not determined.

A particular significance of the results of experiment F is that ADM mediates vasorelaxation partially through increasing production of NO (170). Hence, the ADM-induced vasorelaxation measured in experiments E and G may be partly a consequence of ADM induction of NO within the vessel wall. NO mediates only part of the ADM-induced vasorelaxation in splenic resistance arteries, as L-NMMA only partially inhibited the vasorelaxant response. There are other mechanisms of ADM-induced vasorelaxation that do not involve NO production (170). The NO-independent mechanisms that may account for that part of the ADM-induced vasorelaxation not inhibited by L-NMMA in splenic resistance arteries may relate to production of cAMP (170) (Fig. 9).

EXPERIMENT G: EFFECT OF PHENYLEPHRINE, ENDOTHELIN-1 (ET-1), S-NITROSO-N-ACETYL-D,L-PENICILLAMINE (SNAP) AND ADRENOMEDULLIN (ADM) ON VASOREACTIVITY OF ISOLATED LIPOPOLYSACCHARIDE (LPS)-EXPOSED (150μg/kg/hr for 3 hr *in vivo*) SPLENIC RESISTANCE ARTERIES AND VEINS.

In experiment E, isolated normal splenic resistance vessels were used to measure differential vasoreactivity i.e. they had not been exposed to LPS. By contrast, the vessels used in experiment G were exposed to LPS for a period of 3 hr *in vivo* in order to determine whether the differential vasoreactivity (percent vasoconstriction or vasorelaxation dose-responses) to the vasoactive agents ADM, ET-1 and NO is altered during endotoxemia. Alterations in vascular reactivity to vasoactive agents in both conduit and resistance vessels is a characteristic feature of sepsis (30,224,263). However, not all vascular beds are affected (30,162). The decision to use splenic resistance vessels

that had been exposed to LPS for only a 3 hr period was based on our proposal that the early hemodynamic perturbations during endotoxemia (that arose within the first few hours of LPS exposure) were due, at least in part, to fluid extravasation within the splenic circulation. Hence, there was a need to show that the differential vasoreactivity of splenic resistance arteries and veins to vasoactive agents, which drives the increase in intrasplenic P_C and subsequent intrasplenic fluid efflux, still exists in vessels that have been exposed to LPS. The results of experiment G suggest that there was no significant difference in vasoreactivity between normal and 3 hr LPS-exposed splenic resistance arteries and veins to ADM, ET-1 and NO. Indeed, there was no significant difference in the percent vasoconstriction to ET-1 in terms of the EC_{50} values between unexposed and 3 hr LPS-exposed vessels (Fig. 46; Appendix K) ($p<0.05$), plus the maximal percent vasorelaxation to ADM and NO was not significantly different between unexposed and 3 hr LPS-exposed vessels (Figs. 47 and 48; Appendix L and M) ($p<0.05$).

Changes in vascular reactivity to vasoconstrictor and vasodilator agents are a characteristic feature of endotoxemia and septic shock (30,224,263). The absolute tension (in mN/mm of force) generated by splenic resistance vessels to the highest dose of phenylephrine ($1x10^{-3}$ M) was significantly greater in vessels that had not been exposed to LPS (Fig. 44; Appendix I) ($p<0.05$), although in terms of the percent vasoconstriction or EC_{80} value there was no significant difference between unexposed and 3 hr LPS-exposed vessels (Fig. 45; Appendix J) ($p>0.05$). However, the dose-response curves showing the percent vasorelaxation (with SNAP and ADM) and vasoconstriction (with ET-1) were similar in both unexposed and 3 hr LPS-exposed vessels.

It is surprising that no changes in vascular reactivity to ADM, ET-1 and NO were evident in the 3 hr LPS-exposed splenic resistance vessels given that altered vascular responses is a characteristic feature of endotoxemia and septic shock. However, the absence of such changes in vascular reactivity is not unique to the splenic vasculature as vascular beds throughout the circulatory system respond differently during endotoxemia (30,162).

CHAPTER 5.

CONCLUSION

Based on the results presented in this thesis and their interpretation in the discussion, the following conclusions are made :

1. Splenectomy abolishes the hypotension, hemoconcentration and hypovolemia that typically occur following infusion of LPS into the bloodstream.
2. Splenic denervation exaggerates the intrasplenic fluid efflux, hypotension, hemoconcentration and hypovolemia that typically occur following infusion of LPS into the bloodstream. Hence, SSNA normally limits intrasplenic fluid efflux.
3. ADM and NO increase intrasplenic fluid efflux.
4. ADM increases intrasplenic P_C , due to the differential vasoreactivity of splenic resistance arteries and veins to this vasoactive factor.
5. Splenic resistance vessels (both normal and LPS-exposed) show differential vascular reactivity to ADM, ANF, ET-1, and NO, which are all vasoactive factors that increase in the plasma in response to LPS in the bloodstream.
6. The early hemodynamic perturbations in plasma volume and MAP following infusion of LPS are due, at least in part, to intrasplenic fluid efflux of iso-oncotic fluid.

Figure 34 provides a general overview of the proposed mechanism of intrasplenic fluid efflux and the factors that control it.

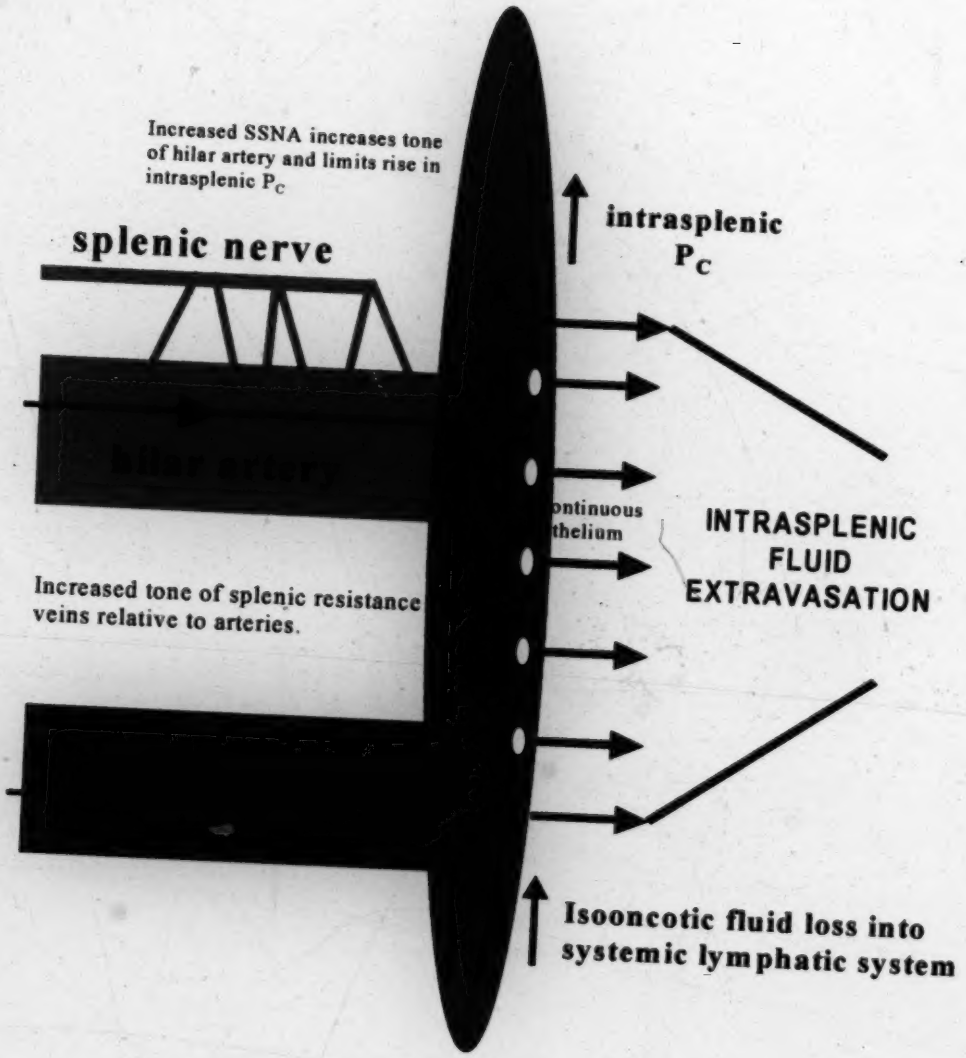
Increased SSNA increases tone of hilar artery and limits rise in intrasplenic P_C
splenic nerve
intrasplenic P_C
hilar artery
ontinuous thelium
INTRASPLENIC FLUID EXTRAVASATION
Increased tone of splenic resistance veins relative to arteries.
Isooncotic fluid loss into systemic lymphatic system

Figure 34. Proposed mechanism for extravasation of protein-rich fluid from the intravascular compartment into the systemic lymphatic system within the splenic circulation.

Methodological considerations and limitations

There are interspecies differences in the anatomy and physiological function of the spleen (199). This does pose difficulties in the applicability of the findings across studies using different animal models. The rationale behind the rat model of endotoxemia used in experiments A and B has been discussed previously (section 1.7). In addition, our use of surgically-recovered conscious unrestrained rats in experiments A and B enabled the hemodynamic measurements to be uncomplicated by the effects of anesthesia and surgical trauma (28,46).

A major difficulty in interpreting much of the data from animal studies is that the doses of LPS that have been used are very large (5-10 mg/kg), the doses have been administered as bolus infusions, and various different animal models have been employed (80,257). Animal studies using large bolus dose infusion of endotoxin invariably report a high mortality rate, with the death of animals occurring only hours after receiving LPS, which clearly suggests that the infusion caused massive vascular and tissue damage. In many instances, studies have utilized anesthetized animal preparations. Even in clinical studies there is a difficulty in interpreting human data (38), as patients have typically received medical treatment and have progressed far along the cascade of events leading to septic shock (118,217,244). Consequently, given these drawbacks, it has been difficult to extrapolate a clear time course of hemodynamic events during endotoxemia or septic shock. Attempts have been made to address this problem. There are animal studies that have involved the continuous infusion of low dose LPS (81,88) or have initiated septic shock through CLP (249,257).

Our use of the ultrasonic blood flow probes (1RB series, Transonic Systems Inc, Ithaca, NY, USA) (Appendix A) was supported by the validity, feasibility and reliability of this technique in the measurement of volume flow in small vessels (39,109,264), plus the validity of the calculated splenic A-V difference as a measure of fluid efflux from the splenic circulation (46). Furthermore, the anatomical feature of arteriovenous shunts in the spleen (100,102) makes it inappropriate to use other techniques of blood flow measurement such as the microsphere method (19). The major advantages of the ultrasonic flowmeter is that it measures volume flow, it does not disrupt blood flow in vessels, it is relatively insensitive to blood flow disturbances (changes in the pattern of blood flow), and it has a stable zero baseline (39,109,264). Moreover, the ultrasonic flowmeter and blood flow probes have been reported to have an accuracy comparable with other *gold standard* techniques of blood flow measurement (206,256,264). Another primary advantage of using the ultrasonic flow probes in experiment B was that the chronic implantation of the flow probes enabled volume flow measurements to be derived from rats that were fully recovered from surgery, conscious and unrestrained. The use of ultrasonic blood flow probes has been reported in the rat (46,256) and in the cat (264).

Close infusion of vasoactive agents into the splenic artery was an approach used in experiments C and D as it allowed much smaller drug concentrations to be administered, with limited peripheral repercussions. Measurements of splenic A-V difference, intrasplenic P_C , and splenic arterial versus venous hematocrit have been obtained from anesthetized rats (8,135,228) (Kaufman, unpublished observation). The use of a whole

blood perfusion medium within a whole animal preparation (experiment D), as opposed to an isolated crystalloid perfusion medium in an *in* vitro preparation, was perhaps the more physiologically relevant choice for measuring P_C considering that rheological factors (such as blood viscosity) can impact on shear stress and patterns of flow through the spleen.

The double occlusion technique that we used to measure intrasplenic P_C (experiment D) has, in other tissues, provided results that are similar to those obtained using classical methods of capillary pressure assessment, such as the isogravimetric technique (77,107,238). The double occlusion technique offered a simple, precise and rapid approach to determining intrasplenic P_C (8,228).

The wire myograph system was used to assess vascular reactivity in *in vitro* experiments. This technique, which enables measurement of isometric tension and attempts to mimic the vessel tension that is seen in *in vivo* conditions, has been used extensively to characterize the vasoreactivity of both small resistance arteries and veins (1,4,8). The advantages and disadvantages of this technique have been reported (74). In brief, the advantages include: the relative simplicity of the experimental technique and apparatus, and the ability to measure isometric tension in small resistance vessels. The disadvantages arising from this technique include: an anatomical distortion of the mounted vessels, damage done to the endothelium and VSMCs due to wire cannulation of a vessel, lack of exposure of blood vessel to normal physiological stimuli such as blood flow, and altered responses to vasoactive agents when compared with studies done

in using the pressure myograph. Reproduction of the responses of splenic resistance vessels in an alternative experimental preparation, such as the pressure myograph, may partially address these concerns.

The concentration range for a vasoactive agent in the *in* vitro studies bracketed the range of reported plasma levels of that factor during endotoxemia in the rat, or was similar to the concentration known to cause increased intrasplenic fluid efflux. Whether the concentrations of vasoactive agent to which the isolated splenic resistance vessels were exposed, had relevance to the effective tissue level of that factor in the body during endotoxemia, is a contentious issue. For example, ET-1 is a vasoactive factor that has an autocrine/paracrine mode of release (120,219). Therefore, the tissue levels of ET-1 may be much higher than the reported plasma levels of this vasoactive factor.

Possible future experiments

The sympathetic nervous system and the renin-angiotension-aldosterone system (RAAS) are crucial parts of the cardiovascular regulatory system that are activated in response to a fall in plasma volume and MAP (82,172). Experiment B provides evidence for the proposal that SSNA limits fluid volume loss from the intravascular compartment. Considering that the sympathetic nervous system typically works in unison with angiotensin II to raise (or maintain) plasma volume and MAP, then the importance of the RAAS in limiting intrasplenic fluid efflux should be considered. In much the same way as increased RAAS activity limits glomerular filtration rate in the kidney, perhaps increased RAAS activation during endotoxemia may limit extravasation of protein-rich

fluid from the intravascular compartment within the splenic circulation. Hence, changes in intrasplenic P_C in response to intrasplenic infusion of angiotensin II should be measured. The differential vasoreactivity of splenic resistance arteries and veins to angiotensin II, using the wire myograph technique, should also be assessed. Considering the physiological functions of angiotensin II, the expected outcome of each of these experiments would be that angiotensin II would induce a reduction in intrasplenic P_C, secondary to a greater vasoconstrictor potency of angiotensin II on splenic resistance arteries than veins.

Another feature of intrasplenic fluid efflux that should be addressed in the future relates to myogenic tone and the degree to which autoregulatory mechanisms control the vascular tone of splenic resistance arteries and veins. Myogenic reactivity in a blood vessel is defined as the ability of the vascular smooth muscle to contract in response to stretch or an increase in transmural pressure (60,126,127). We could speculate that increased pressure within the venous side of the splenic circulation could result in myogenic vasoconstriction of splenic resistance veins, causing intrasplenic P_C to rise and intrasplenic fluid efflux to increase. Indeed, the strong myogenic response that occurs in the hepatic portal vein (185,214), which receives venous blood from the splenic circulation, could also exist in the splenic resistance veins. A pressure myograph system, a technique that enables transmural pressure and blood flow in an isolated blood vessel to be altered independently, could be used to investigate the myogenic response in splenic resistance arteries and veins. Hence, alongside the evidence already gathered for the

nervous and hormonal control of intrasplenic fluid efflux, the importance of myogenic reactivity could also be considered.

In conclusion, the results of experiments A-G provide support for a role of the spleen in the control of plasma volume and blood pressure during the early hemodynamic perturbations induced by LPS. It is established that the spleen has immunological and hematological functions. Given the results of the experiments that comprise this thesis, regulation of plasma volume and blood pressure during physiological and pathophysiological circumstances can now also be ascribed to this organ. The clinical significance of the findings reported in this thesis may reside in: i) the fact that extravasation of protein-rich fluid from the splenic circulation (and other vascular beds with a discontinuous endothelium) could contribute to the early hemodynamic changes observed in human patients with septic shock, and ii) understanding the physiological significance of intrasplenic fluid efflux, in the context that this phenomena contributes to circulatory insufficiency during septic shock but is also a mechanism for recirculation of immune cells.

CHAPTER 6.

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CHAPTER 7.

APPENDICES

APPENDIX A

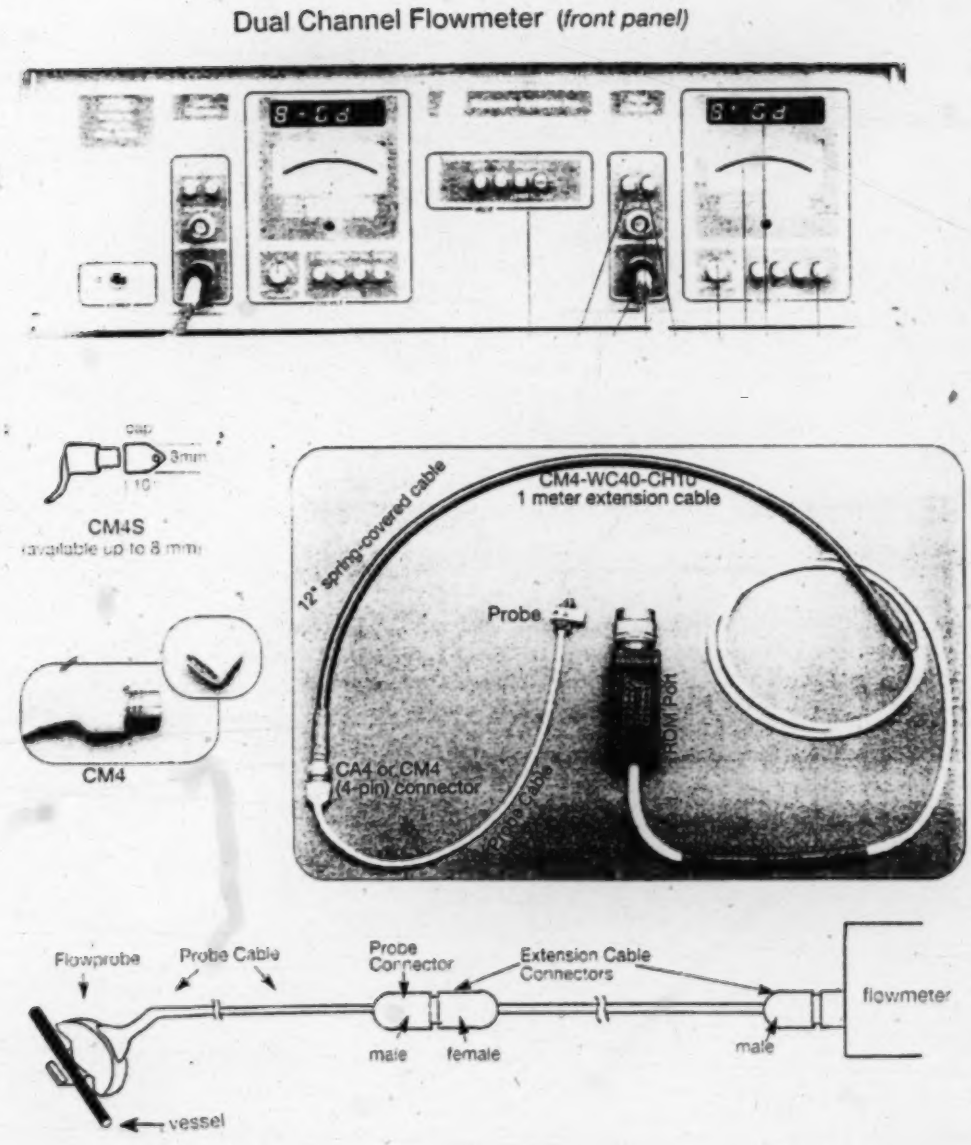

Figure 35. Apparatus used for splenic blood flow recordings in conscious unrestrained rats (experiment B).

The chronic implantation of blood flow probes around the splenic artery and vein of a rat was originally reported by Chen and Kaufman (1996) (46). Chronic implantation of the blood flow probes in experiment B allowed the measurement of blood flow to the spleen of a conscious unrestrained rat during continuous intravenous infusion of LPS. An estimation of the extravasation from the splenic circulation was derived through the comparison of splenic arterial versus venous blood flow. It was possible to obtain a continuous recording of blood flows in the splenic artery and vein, in a manner where vessels were fully functional for flow measurement in a non-constrictive and non-occlusive design. The validity and reliability of the blood flow probes has been confirmed by others (59,109,255).

For an extensive description of the T206 flowmeter and 1RB series blood flow probes (used in experiment B) the following sources are provided :

- Transonic T206 animal research flowmeter manual. *Transonic Systems Inc*. Review. 3/31/92.
- Fundamental techniques for hemodynamic studies in the rat. *Transonic Systems Inc.* 10th Anniversary Special Edition. (1993)
- Flowmeters for medicine and research catalogue. *Transonic Systems Inc*. Product Catalogue. 1994-1995.

APPENDIX B

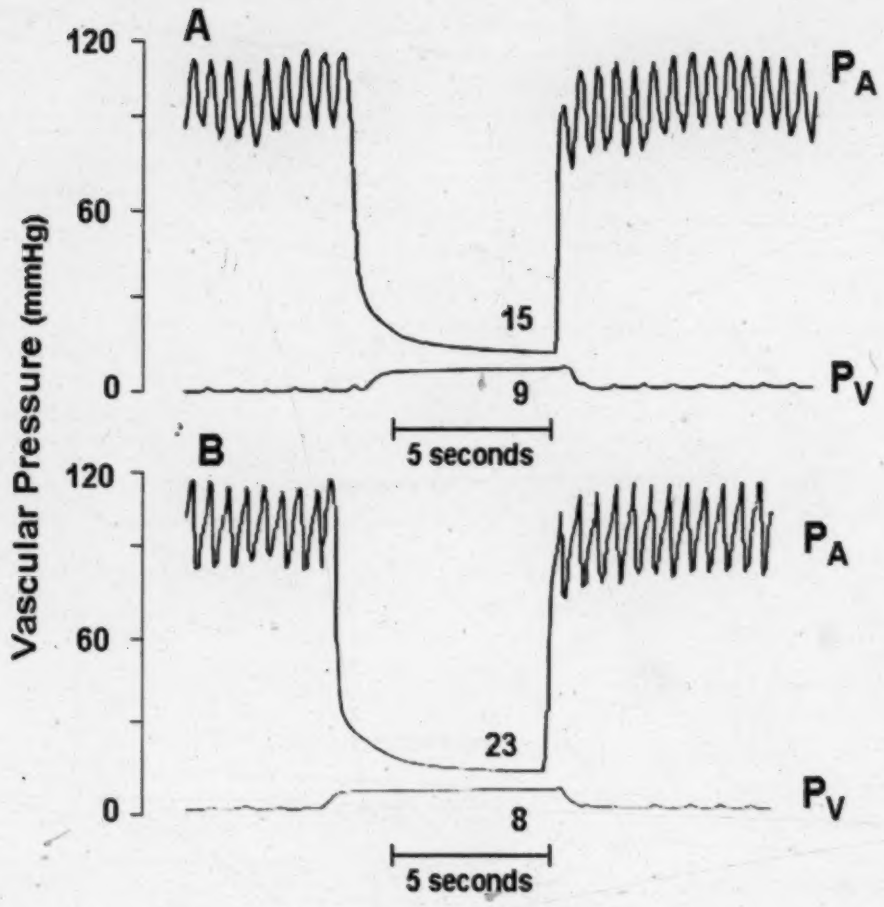

Figure 36. Double vascular occlusion technique for determining intrasplenic P_C (experiment D). An example of the pressure tracing for arterial pressure (P_A) and venous pressure (P_V) during the double occlusion in an isolated, blood-perfused spleen in A) saline-infused control and B) adrenomedullin (ADM)-infused (ADM concentration was infused at a dose of 9 ng/ml into the splenic artery) rat.

APPENDIX C

INTERNAL CIRCUMFERENCE: TENSION CHARACTERISTICS

Preliminary experiments were initially completed to confirm the optimal setting for splenic resistance arteries and veins (experiments E and G). Tension developed is due to both active and passive properties of the vessel wall. The passive tension is due to the elastic properties of the vessel wall, whereas active tension is due to the activity of the contractile properties of the VSMC (147). The active tension that is generated by the VSMC is directly related to the degree of overlap between actin and myosin filaments (130). When the filaments are too far apart, there is no overlap of the actin and myosin filaments, resulting in no cross-bridge formation. When the filaments are at an optimal length, the number of cross-bridge associations is increased and maximum tension can be generated. However, when the muscle filaments are compressed (or too close together) the actin filaments interfere with one another. This causes the tension developed to be less than maximal (194), because the cross-bridging is not optimal.

PROTOCOL FOR DETERMINATION OF OPTIMAL SETTING FOR SPLENIC RESISTANCE VESSELS

- Mount artery (or vein) and set at zero tension (wires touching) (Fig. 38)
- Warm up artery (or vein) for 30 minutes in HEPES-PSS buffer, changing HEPES-PSS every 10 minutes

TOTAL CURVE:

- Replace HEPES-PSS with potassium chloride-depolarizing solution (140 mmol/L)

- Adjust micrometer in step-wise increments one notch (25μm) at a time (~9-10 notches in total) and record the diameter and tension at each point
- Reset vessel back to starting point
- Remove potassium chloride-depolarizing solution and replace with HEPES-PSS
- 30 minute wash-out period (change HEPES-PSS every 10 minutes)

PASSIVE CURVE:

- Replace HEPES-PSS with calcium-free buffer with papaverine (10^{-4} mol/L)
- Incubate artery (or vein) for 20 minutes (do not change buffer)
- Adjust micrometer in step-wise increments and record the diameter and tension at each point (same number of adjustments as the total curve)

Calculations: (example shown on Table 1 and Fig. 37)

- Prepare graph (Fig. 37):

  Total curve

  Passive curve

  Active curve = Total – Passive

- From graph chose internal circumference that produced the least passive tension and the most active tension (Fig. 37)
- Input paired data set of diameter and tension from passive curve into computer program (Table 1)
- Computer program uses the Law of LaPlace to calculate the point on the passive curve that corresponds to an effective transmural pressure of 100mmHg (hilar artery) **(Length @ 100mmHg)** (or 5mmHg for hilar vein; **Length @ 5mmHg**)
- This value is called the L_{100} (artery) (or L_5 for veins)

- Program also gives you the length at each point of the passive curve (**Length** column)
- In the **Displacement** column the diameter that was chosen (200 μm), to produce the most active tension and the least active tension, is divided by the **Length @ 100mmHg (artery)** (or **Length @ 5mmHg for vein)**
- This value will be the percent of the L_{100} (artery) (or L_5 for a vein) that is required to give you an optimal setting
- In this example the optimal setting is 65% of L_{100}; calculated from displacement giving the most active tension and the least active tension divided by the **Length @ 100mmHg (artery)** 528.5/813.5=65%
- Our preliminary experiments on arteries resulted in an optimal setting of 65 ± 5% of L_{100} (n=10). Preliminary experiments for veins resulted in an optimal setting of 80 ± 3% of L_5 (n=9). Below is an example of the tension characteristics data for an artery that was used to determine the optimal tension setting.

Table 1. Internal Circumference: Tension Characteristics – Computer Program

Displacement	Force	Length	Tension	LaPlaceT@100mmHg
125	0.031	378.5	0.076	0.803
150	0.049	428.5	0.120	0.909
175	0.080	478.5	0.196	1.015
200	**0.110**	**528.5**	**0.269**	**1.121**
225	0.140	578.5	0.343	1.227
250	0.180	628.5	0.441	1.333

Length @ 100 mmHg = 813.5

Displacement @ 100 mmHg = 342.5

.65L = 528.8

Displacement @ .65L = 200.1

Force @ .65L = 9.9×10^{-2} grams

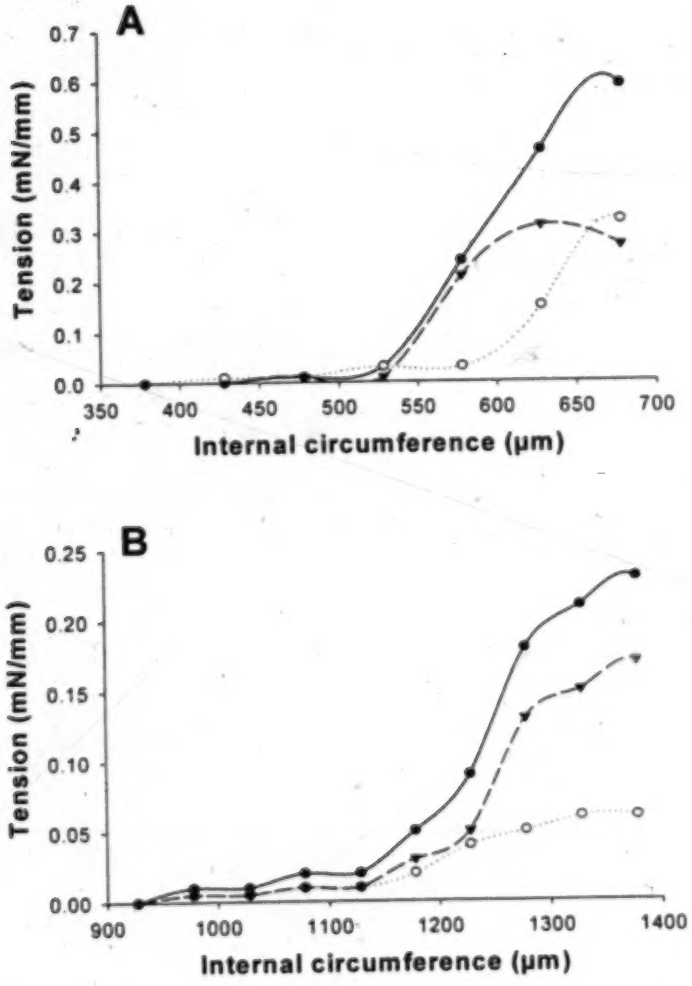

Figure 37. Typical passive-active tension characteristics curves for A) hilar artery and B) hilar vein. The optimal tension settings for each vessel type were the internal circumference that produced maximal active tension and least passive tension. The total (closed circles), active (closed triangles) and passive (open circles) curves are illustrated.

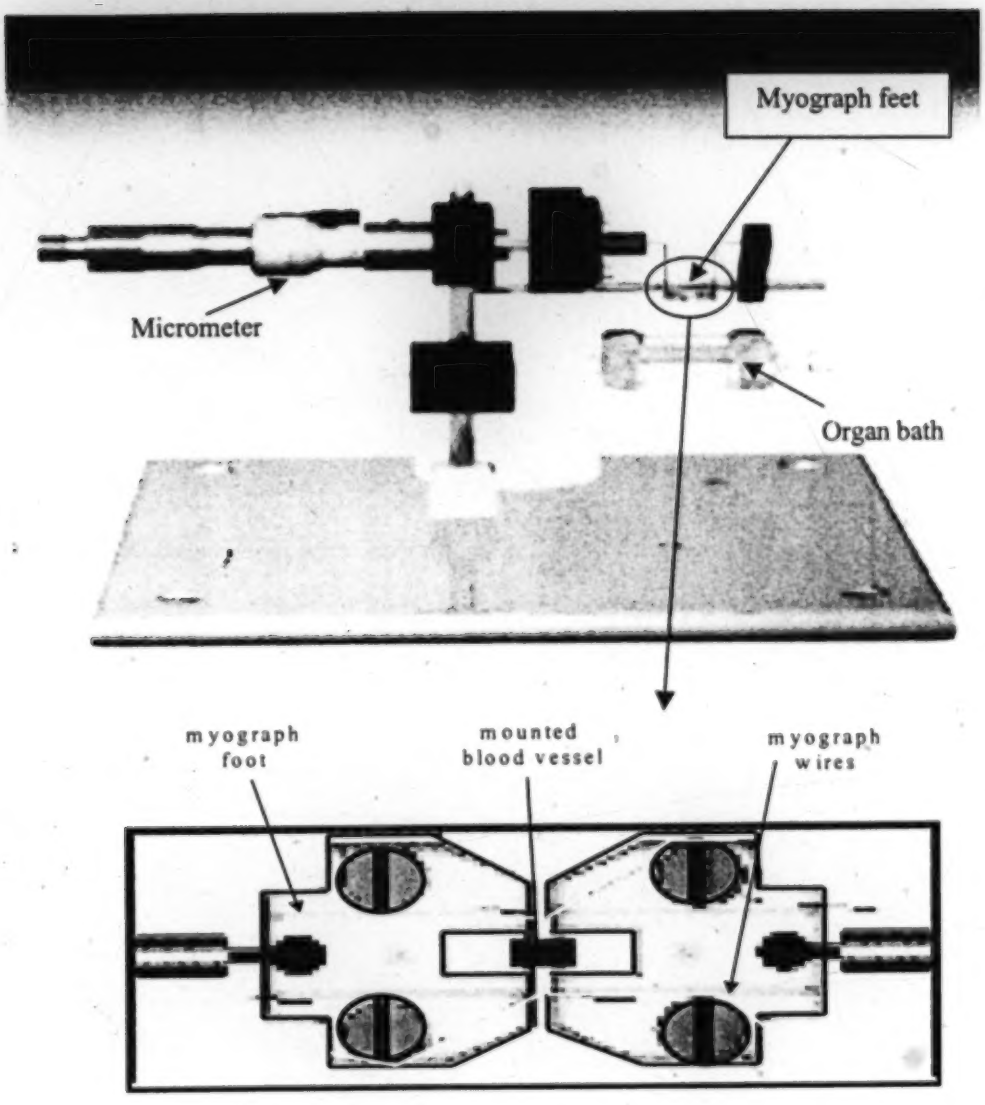

Figure 38. Illustration of the wire myograph apparatus.

APPENDIX D

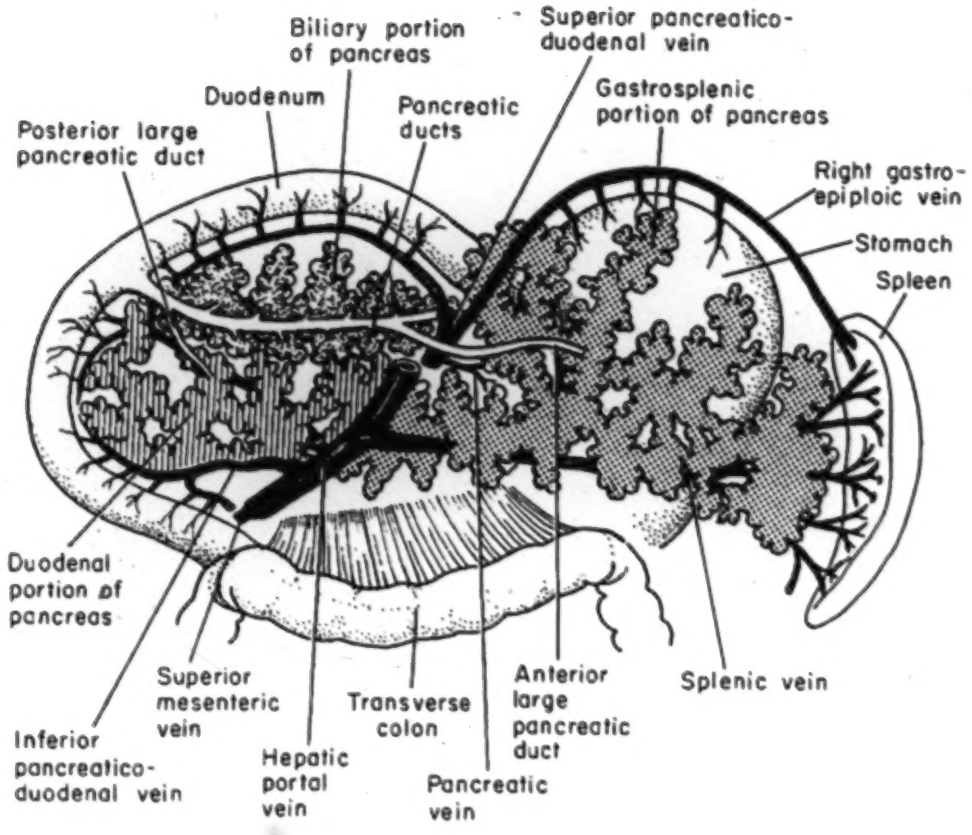

Figure 39. The spleen and its associated anatomical relationships. Reproduced from Waynforth and Flecknell (1992) (251).

APPENDIX E

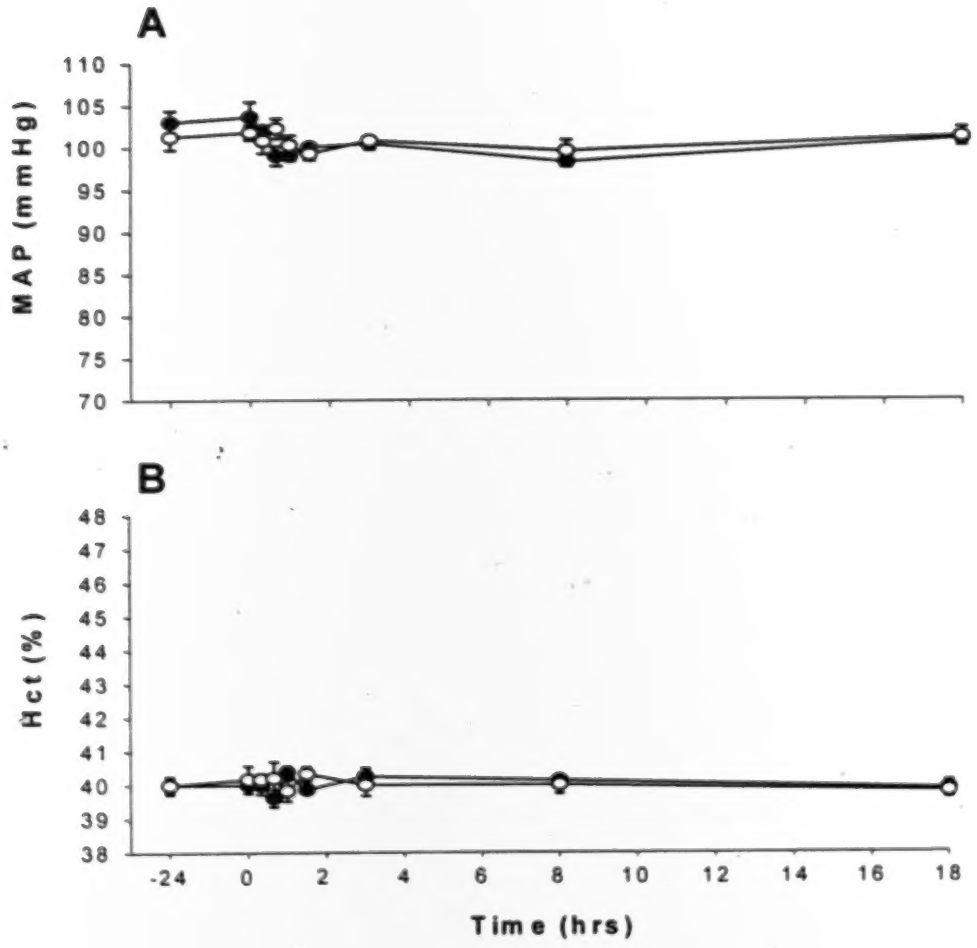

Figure 40. A) Mean arterial pressure (MAP) and B) hematocrit (Hct) during intravenous infusion of saline in intact (solid circles, n=8) and splenic denervated (open circles, n=8) conscious rats. Vertical bars delineate standard error of the mean. Two-way repeated measures ANOVA plus Student Newman-Keuls test for multiple comparisons.

APPENDIX F

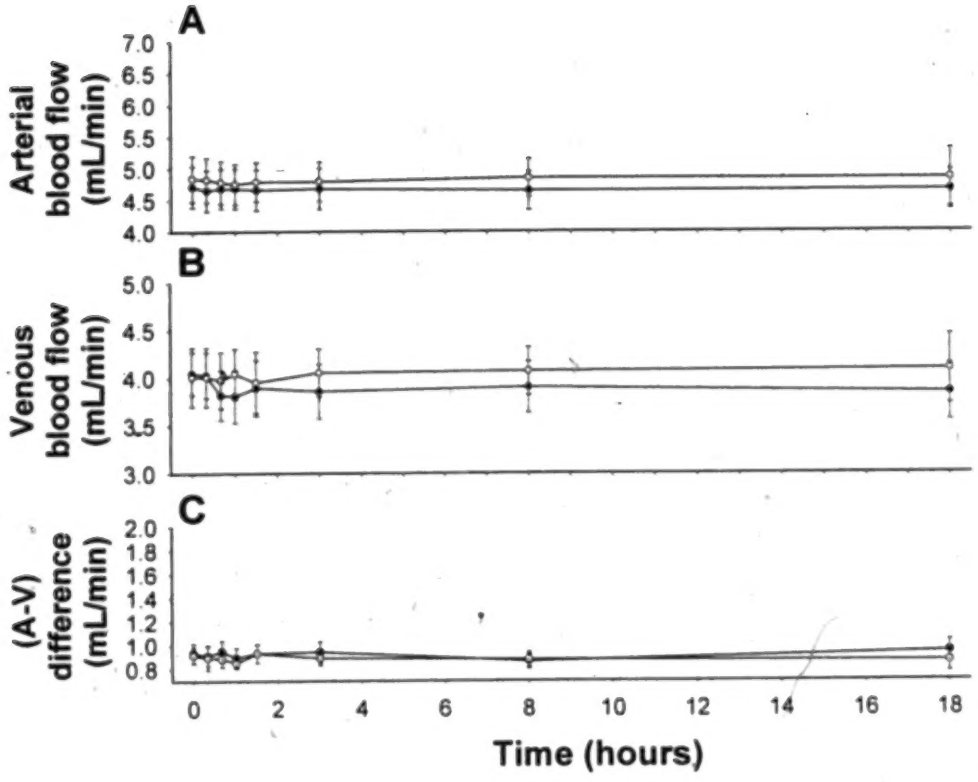

Figure 41. Splenic A) arterial and B) venous blood flows in denervated (open circles, n=10) and intact (solid circles, n=8) conscious rats during saline infusion, and C) fluid efflux (A-V difference) in denervated (open circles, n=8) and intact (solid circles, n=8) groups. Vertical bars delineate standard error of the mean. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

APPENDIX G

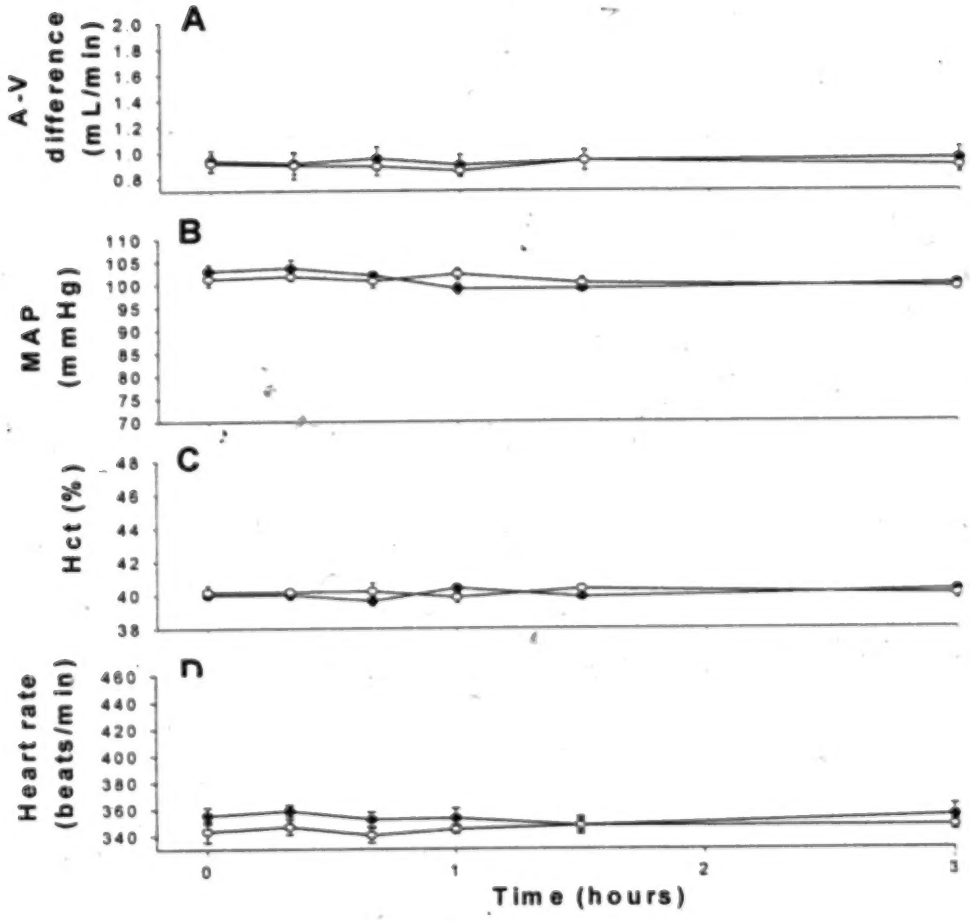

Figure 42. A) Splenic (A-V) difference B) mean arterial pressure (MAP) C) hematocrit (Hct) and D) heart rate during intravenous infusion of saline in intact (solid circles) and splenic denervated (open circles) conscious rats. Vertical bars delineate standard error of the mean. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

APPENDIX H

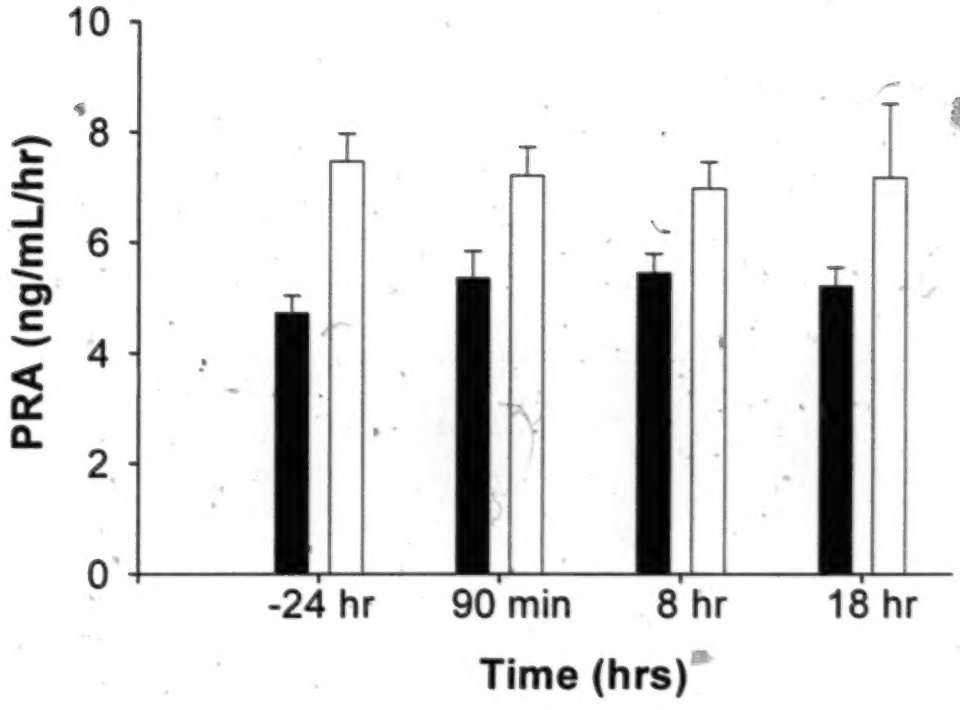

Figure 43. Plasma renin activity (PRA) before (-24 hr) and during intravenous infusion of saline in intact (closed bars, n=7) and splenic denervated (open bars, n=7) conscious rats. Vertical bars delineate standard error of the mean. Two-way repeated measures ANOVA plus Student-Newman Keuls test for multiple comparisons.

APPENDIX I

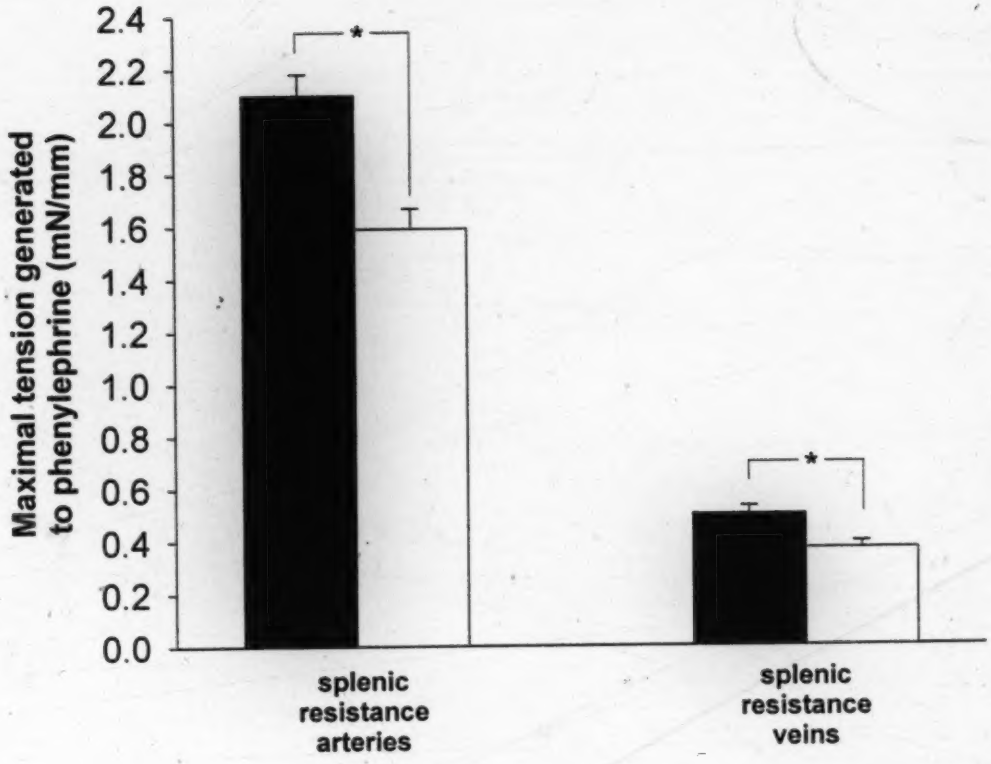

Figure 44. Maximal tension generated in unexposed (closed bars) and 3 hr LPS-exposed (open bars) splenic resistance vessels at the highest concentration of phenylephrine (10^{-3} M) used in constructing a preconstriction curve for each vessel. Vertical bars delineate standard error of the mean. *, Significant difference between unexposed and 3 hr LPS-exposed splenic resistance vessels. Unpaired Student's t-test.

APPENDIX J

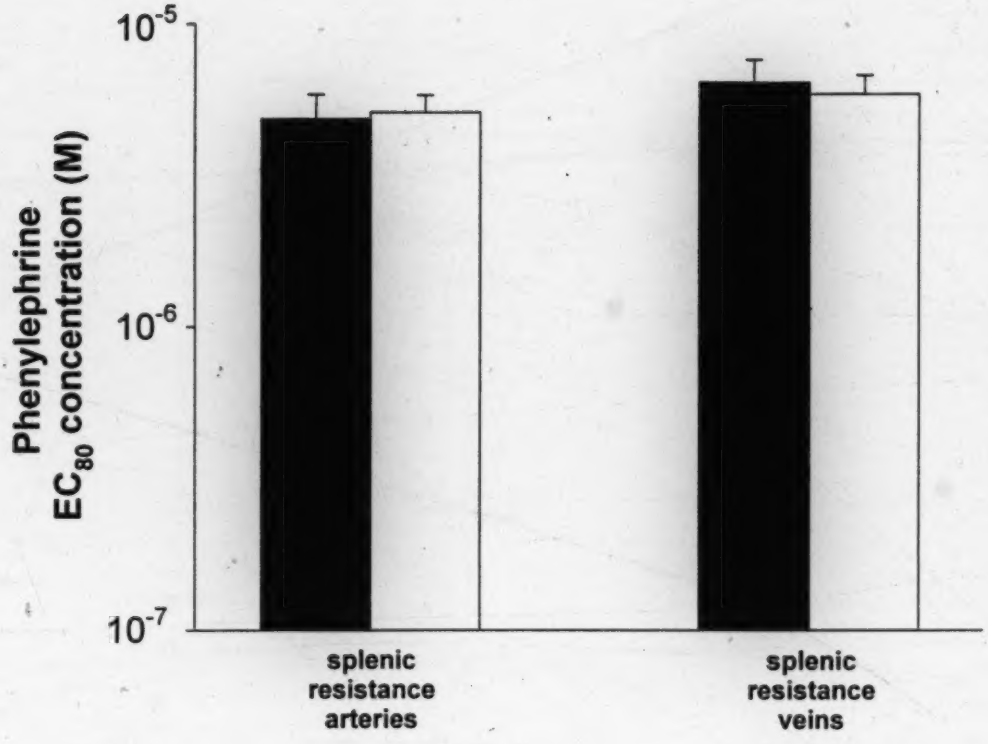

Figure 45. EC_{80} concentration values for phenylephrine preconstriction in unexposed (closed bars) and 3 hr LPS-exposed (open bars) splenic resistance vessels. Vertical bars delineate standard error of the mean. One-way ANOVA on ranks plus Dunn's test for multiple comparisons.

APPENDIX K

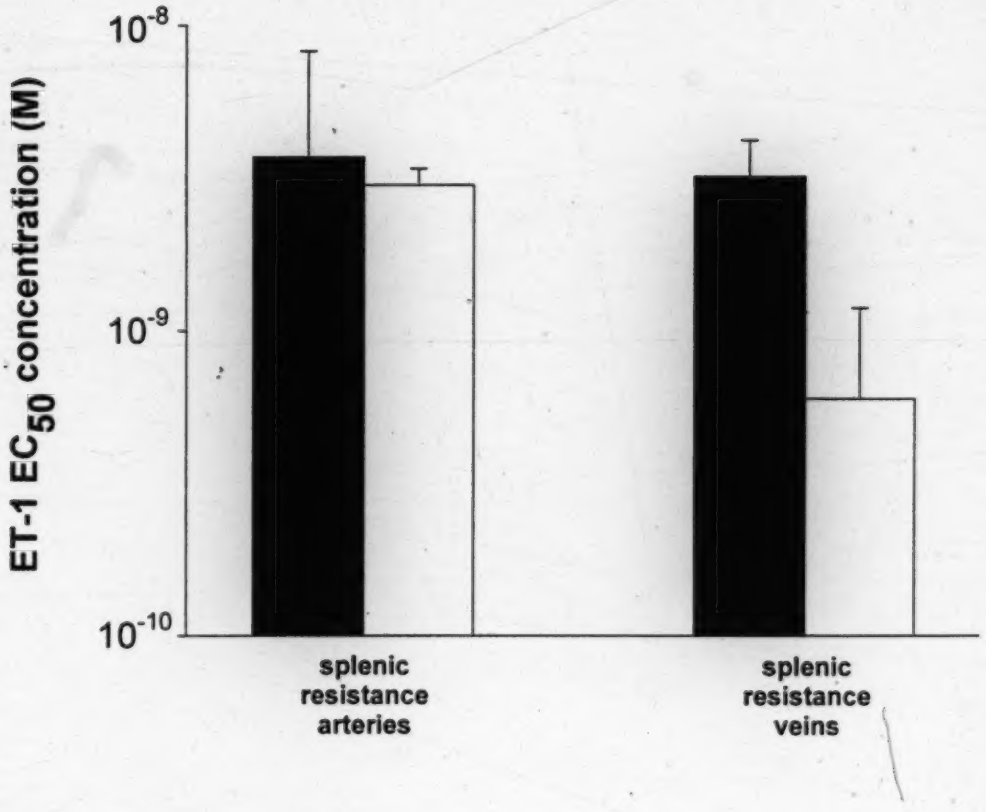

Figure 46. EC_{50} concentration values for endothelin-1 (ET-1) vasoconstriction in unexposed (closed bars) and 3 hr LPS-exposed (open bars) splenic resistance vessels. Vertical bars delineate standard error of the mean. Mann-Whitney rank sum test.

APPENDIX L

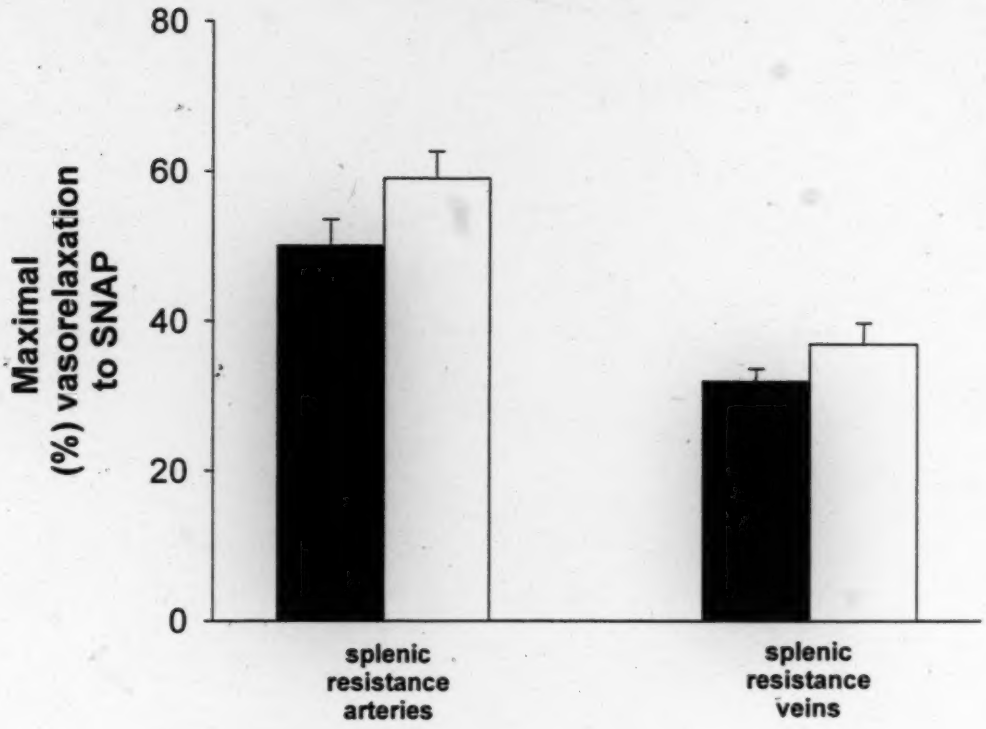

Figure 47. Maximal percent vasorelaxation to S-nitroso-n-acetyl-D,L-penicillamine (SNAP) in unexposed (closed bars) and 3 hr LPS-exposed (open bars) splenic resistance vessels. Vertical bars delineate standard error of the mean. One-way ANOVA plus Dunn's test for multiple comparisons.

APPENDIX M

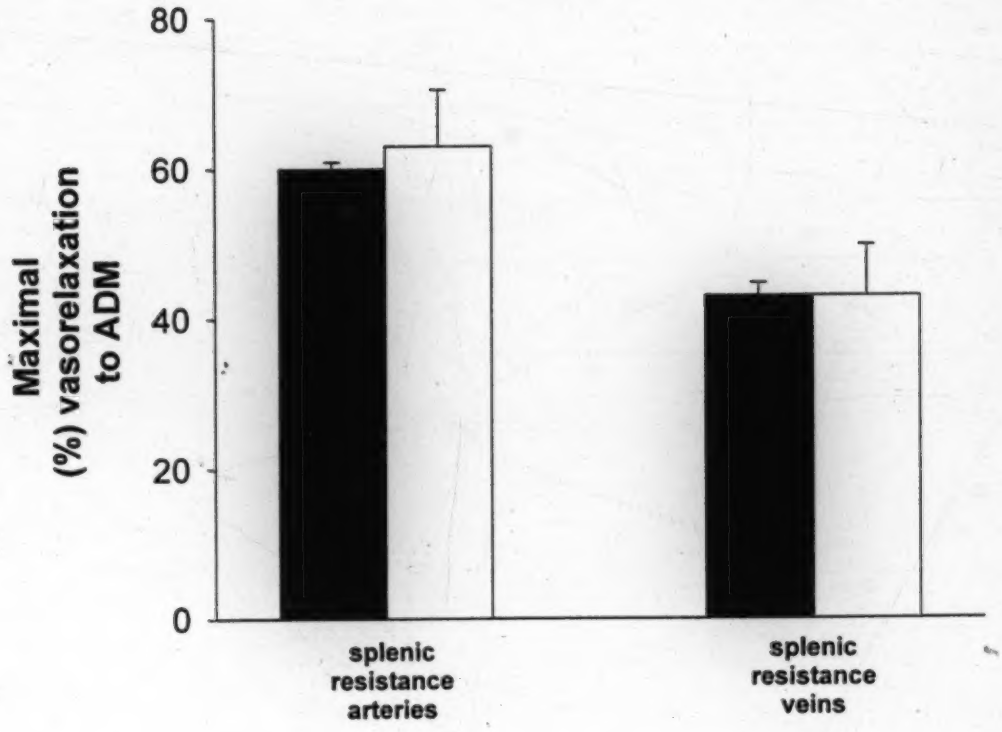

Figure 48. Maximal percent vasorelaxation to adrenomedullin (ADM) in unexposed (closed bars) and 3 hr LPS-exposed (open bars) splenic resistance vessels. Vertical bars delineate standard error of the mean. One-way ANOVA plus Dunn's test for multiple comparisons.